University of Reading

Department of Food and Nutritional Sciences

The Flavour of Cooked Cheese

Thesis submitted for the requirement for the degree of Doctor of Philosophy in Food & Nutritional Science

By

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Abstract

Cheese is a popular ingredient in cooked dishes (such as pizza, toppings on baked dishes and in ready-meal sauces) and is an important commodity for the UK dairy industry. Fat contributes a large percentage of the composition of cheeses, leading some consumers to have health concerns regarding consumption of cheese. The production of reduced-fat variants which function comparably to full-fat cheeses in cooked applications is a focus for the dairy industry. However, little information is available in published literature on the compounds responsible for the flavour of cooked cheese, the volatile changes which occur when cheese is cooked, or the effect of reducing-fat on cooked cheese flavour.

The first aim of this research was to characterise cooked cheese flavour, including volatiles (especially odorants) and selected non-volatiles (tastants and precursors). Additionally, this work aimed to investigate the role of fat in development of cooked cheese flavour.

Using solid phase microextraction (SPME) the volatile profiles of six cooked cheeses (mozzarella, Parmesan, mature Cheddar, mild high-fat Cheddar, mild medium-fat Cheddar, mild low-fat Cheddar) were characterised and compared to their uncooked counterparts (chapter three). Fatty acids and esters decreased in concentration during cooking, while many other volatile classes including 2-methylketones, pyrazines, Strecker aldehydes, lipid-derived aldehydes and furanones increased during cooking.

GC-O was performed on the mature Cheddar using SPME, which identified odorants responsible for cooked cheese flavour. Many odorants in cooked cheese (including Strecker aldehydes, furanones, sulfur compounds, fatty acids and 2-methylketones) have been detected previously in uncooked cheese, but were significantly ($p < 0.05$)

higher in concentration in cooked cheeses. Others ((3-methyl-2-butene-1-thiol, (furan-2-yl)methanethiol, cyclotene, 3-ethyl-2,5-dimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, 2-methyl-3-methyldithiofuran and (E)-2-decenal) have not been reported previously as odorants in uncooked Cheddar.

A solvent assisted flavour evaporation (SAFE) approach for comparison of matrices with substantially differing fat contents was developed (chapter five) and used during the solvent extraction and SAFE of cooked mild high (HF), medium (MF) and low-fat (LF) cooked Cheddars (chapter six). GC-O was performed on SAFE extracts from HF and LF cooked Cheddars and compared to determine the role of fat in cooked cheese flavour. Far fewer odorants were detected in the LF cheese than the HF, which reflects the lower concentration of the majority of volatiles in the LF cooked Cheddar. Comparison of SPME and SAFE data showed similar trends for comparison of HF, MF and LF cheese.

Selected non-volatiles were quantified in the six cheeses, both uncooked and cooked (chapter four). Amino acids, sugars and γ-glutamyl-dipeptides all decreased in concentration during cooking, which is consistent with their participation in the Maillard reaction. Diketopiperazines (DKPs) increased in concentration during cooking and were above their taste threshold in some cooked cheeses while below threshold in uncooked cheeses. In particular, more extensively aged cheeses formed more DKPs when cooked than younger cheeses, which is likely to be due to the formation of DKP precursors during maturation. As DKPs are bitter and metallic, they may contribute to bitter flavour in cooked aged cheeses. Scanning electron microscopy (SEM) on the cooked Cheddars confirmed substantial structural differences (chapter six), which may contribute to the differences in generation of flavour.

Acknowledgement

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To my family. You fostered in me the curiosity which allows me to be a scientist, and of course a lifelong love of food. Without either, this thesis would certainly not have been possible. To my mother, I am sorry that I never managed to ‘sneak a joke in’ – maybe in my next thesis?

To my friends. Your companionship, encouragement and laughter have kept me going through the hardest times, and I can think of no one better with whom to celebrate my success.

To Oliver. For your consistent, quiet, assured belief in me, for lending me your calm whenever I needed it and for about a million glasses of squash, I thank you from the bottom of my heart.

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Abbreviations

AEDA	Aroma extract dilution analysis
ANOVA	Analysis of variance
Ched	Mature Cheddar
CLSM	Confocal laser scanning microscopy
DHS	Dynamic headspace
DKP	Diketopiperazine
FD	Flavour dilution
FFA	Free fatty acid
GC	Gas chromatography
GC-MS	Gas chromatography-mass sprectrometry
GC-O	Gas chromatography-olfactometry
HF	High fat mild Cheddar
HF24	HF sample aged for 24 months
HPLC	High performance liquid chromatography
HSD	Honest significant difference
HS-SPME	Headspace-solid phase microextraction
LC	Liquid chromatography
LC-MS	Liquid chromatography- mass spectrometry
LC-MS-MS	Liquid chromatography-tandem mass spectrometry
LF	Low fat mild Cheddar
LRI	Linear retention index
LSD	Least significant difference
MF	Medium fat mild Cheddar

Mozz	Mozzarella
MRM	Multiple reaction monitoring
MSD	Mass spectrometry detector
Parm	Parmesan
PC	Principal component
PCA	Principal component analysis
SAFE	Solvent assisted flavour evaporation
SDE	Simultaneous distillation extraction
SEM	Scanning electron microscopy
SHS	Static headspace
SIDA	Stable isotop dilution assay
SPME	Solid phase microextraction
TGSC	The good scent company
TLHVD	Thin layer high vacuum distillation
WSE	Water soluble extract

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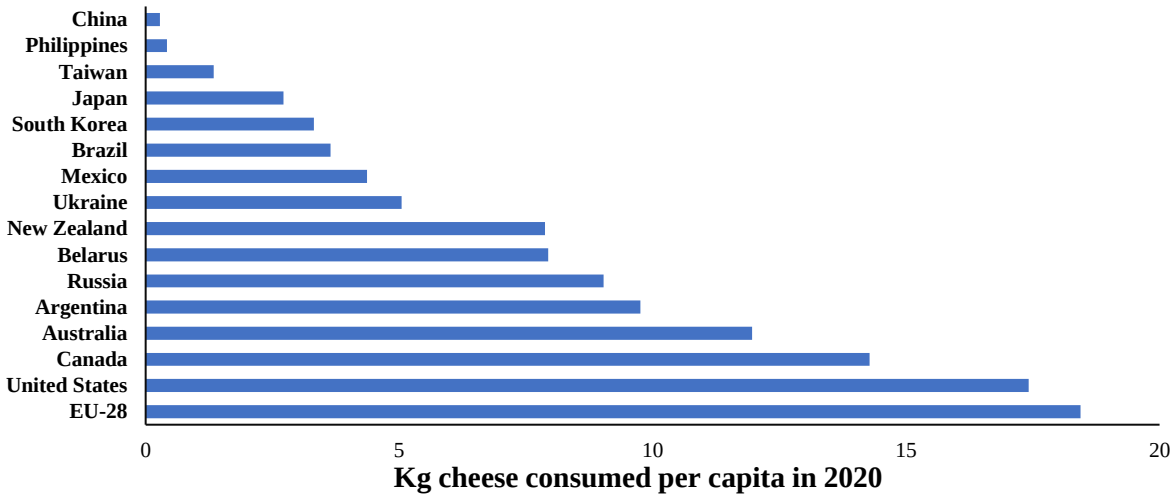
Chapter 1 – Introduction, Aims and Hypotheses

1.1 Introduction

Cheese has been a fundamental ingredient in cooked dishes for millennia. In one of the earliest written references to cooked cheese, the ancient Greek poet Antiphanes described the cooking of *plakous* (a baked dish including wheat flour, goats' cheese and honey) in the 4th century B.C (Goldstein, 2015). In the modern day, cooked cheese remains highly popular for its flavour and melt properties in dishes including pizza, various pasta (e.g. macaroni cheese), fondue, toasted sandwiches, and grilled cheese (e.g. halloumi).

Cheese is a major commodity for the global dairy industry. The value of the cheese industry is estimated to reach 105 billion US dollars globally by 2026 (M. Shahbandeh, 2021). It is especially important to the economies of European countries, as the EU is the largest exporter of cheese globally (Augere-Granier, 2018).

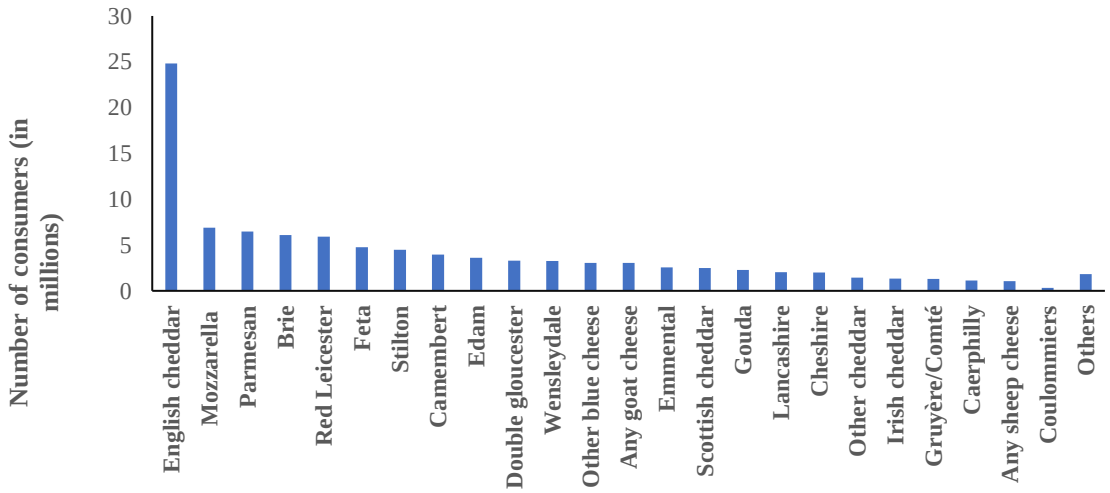
Figure 1.1 Cheese consumption in 2020 per capita, by country.



(CLAL., 2021)

Cheese consumption is highest in the EU (including UK), United States, Canada and Australia (see figure 1.1) (CLAL., 2021). Within these markets, cheese is consumed very frequently, for example 85% of UK consumers consume cheese at least once weekly (Kantar Media, 2021a). Cheese varieties vary in popularity by country. In the UK the most popular cheeses are Cheddar, mozzarella (both traditional and low-moisture) and Parmesan (see figure 1.2) (Kantar Media, 2021b).

Figure 1.2 Estimated cheese consumption in the UK in 2019 by variety



(Kantar Media, 2021b)

Low-fat cheese represents a relatively small, but growing share of the market, with 21% of UK consumers reporting purchasing low-fat cheese in 2020 (Kantar Media, 2021a. Various flavour and texture challenges have been reported with low-fat cheese (Guinee et al., 2000; Guinee & Kilcawley, 2004; Mistry, 2001; Tunick et al., 1993; Vítová et al., 2007), which may affect its popularity with consumers. Low-fat cheese is produced from part or all skimmed milk (Mistry, 2001). This reduces

saturated fat content in cheese by up to 90%, which is associated with reduced cardiovascular health risks for consumption. Given the global obesity crisis and recent negative publicity regarding saturated fat and dairy consumption, improving the overall quality of low-fat cheese is an important topic for the dairy industry.

While cheeses are often consumed uncooked, it is also very common for consumers to purchase cheese for use as an ingredient in cooked dishes (Keech et al., 2014; Mesías et al., 2003). In the UK, the popularity of Cheddar and mozzarella is likely to be driven by their use in cooked dishes. In a recent consumer study, 77% of Cheddar consumers described purchasing it for use in toasted sandwiches (Bord Bia Insight Centre, 2018). In the ready-made food industry, 49% of ready meals launched in Europe between 2018-2020 contained cheese (Mintel, 2021). Of these, 57% contained mozzarella, most of which were pizzas (Mintel, 2021). In the context of industrial pizza manufacture, mozzarella/Cheddar blends are often used to achieve a cheese topping with desirable melt, stretch and flavour properties (Singh et al., 2003a).

Despite the popularity of cheese as an ingredient in cooked dishes, prior to this research there was very limited data in the literature to describe cooked cheese flavour.

1.2 Research objectives

The aim of this research was to generate understanding of selected key aspects of the flavour of cooked cheese. As little research had been done in this area previously, specific areas of focus were identified at the outset of the project.

The scope of this work includes:

1. Characterisation of the volatiles in cooked cheese, including identification of odorants using gas chromatography olfactometry (GC-O).

2. Quantitation of selected non-volatiles in cooked cheese.

3. Characterisation of the role of fat on both the volatile and non-volatile composition of cooked cheese.

Further details are given in section 2.5.

1.3 Hypotheses

Hypothesis 1: Cooking cheese changes the concentration of flavour compounds responsible for aroma (odorants) and taste (tastants).

Previous literature (Dumont et al., 1976) identified some changes in the volatile compounds present when Gruyère is cooked. This work will explore whether cooking cheese leads to a change in the presence of odorants using GC-O. Furthermore, selected tastants in cheese such as amino acids, γ-glutamyl peptides may be lost due to participation in the Maillard reaction, but may also form from the breakdown of larger peptides.

Hypothesis 2: Fat level in cheese influences the formation of Maillard, lipid and lipid-Maillard interaction derived odorants during cooking.

As lipids contribute a large portion of cheese, and lipid derived flavour is a key aspect of many other cooked foods, this work will investigate the presence of lipid-derived volatiles in cooked cheese. In low-fat cheeses the casein network is less interrupted by fat globules (Mistry, 2001), which may lead to more protein-sugar interactions and formation of Maillard products. This work will investigate the role of fat content on Maillard-derived odorants and will compare low, medium and

high-fat cheese to determine the importance of fat in generation of cooked cheese flavour.

Hypothesis 3: Fat level does not affect the concentration of selected tastants (amino acids, γ-glutamyl peptides, DKPs, organic acids) in cooked cheese.

None of the selected tastants in this study are lipid-derived, so it is likely that cooked LF cheese contains a similar concentration of these tastants to cooked HF cheese.

1.4 Novelty and significance of the research

This thesis will outline the odorants of cooked cheese and describe the role of cooking on cheese tastants. The subject of cooked cheese flavour holds significance for the dairy industry. Understanding the flavour changes which occur during cooking facilitates selection and optimisation of cheeses for cooked applications. Such work has previously been focussed on visual and textural properties, affecting browning and stretch, but has fallen short of considering flavour implications. Furthermore, the contribution of fat to the flavour of cooked cheese is an important topic for the dairy industry, contributing to the improvement of the suitability of reduced fat cheeses for cooked applications.

Three cheeses, Cheddar, mozzarella and Parmesan were selected for study in this project due to their market popularity (see section 1.1) and importance to the cheese industry. Furthermore, these cheeses represent a range of compositional, maturation and manufacturing differences (see section 2.2).

1.5 Thesis outline

This thesis has been written as a series of published, submitted and draft papers, consisting of seven main chapters. **The second chapter** is a literature review outlining relevant literature on uncooked and cooked cheese flavour.

The third chapter is a characterisation of the volatile compounds in six cooked cheeses using solid phase microextraction.

The fourth chapter is an overview of the characterisation of non-volatiles in cooked cheese compared to uncooked cheeses.

Both the third and fourth chapters are being prepared for journal publication.

The fifth chapter outlines the development of a new extraction procedure for comparison of low and high fat matrices using solvent-assisted flavour evaporation.

It has been published in Food Analytical Methods:

Sullivan, R.C., Fagan, C.C. & Parker, J.K. (2021) Improved recovery of higher boiling point volatiles during solvent-assisted flavour evaporation. *Food Anal. Methods*, 14**,** 2486–2493 https://doi.org/10.1007/s12161-021-02074-5

In the sixth chapter the method outlined in chapter five was employed for the comparison of volatiles compounds detected in low and high-fat mild Cheddar by liquid extraction and SAFE, including GC-O.

Chapter six is in the process of preparation for submission for journal publication.

The Seventh chapter presents an overall summary of the research and proposes direction for future work on this topic.

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Chapter 2 – Literature Review

Until this study, very little published work has focussed on cooked cheese aroma, although a handful of relevant studies are discussed in section 2.4.3. In line with the hypotheses of this study, cooked cheese flavour was predicted to maintain aspects of uncooked cheese flavour, but thermally induced reactions such as the Maillard reaction and lipid degradation were also predicted to cause flavour changes in cheese during cooking. As such, this literature review focusses on three main themes:

1. The key compounds responsible for uncooked cheese flavour (section 2.3) and common methods for their characterisation.
2. The processes and products formed by the Maillard reaction and lipid degradation (section 2.4).
3. The processes of cheesemaking and ripening (proteolysis, lipolysis and lactose and citrate metabolism) which affect the development of flavour and flavour precursors in cheese, (sections 2.1 and 2.2).

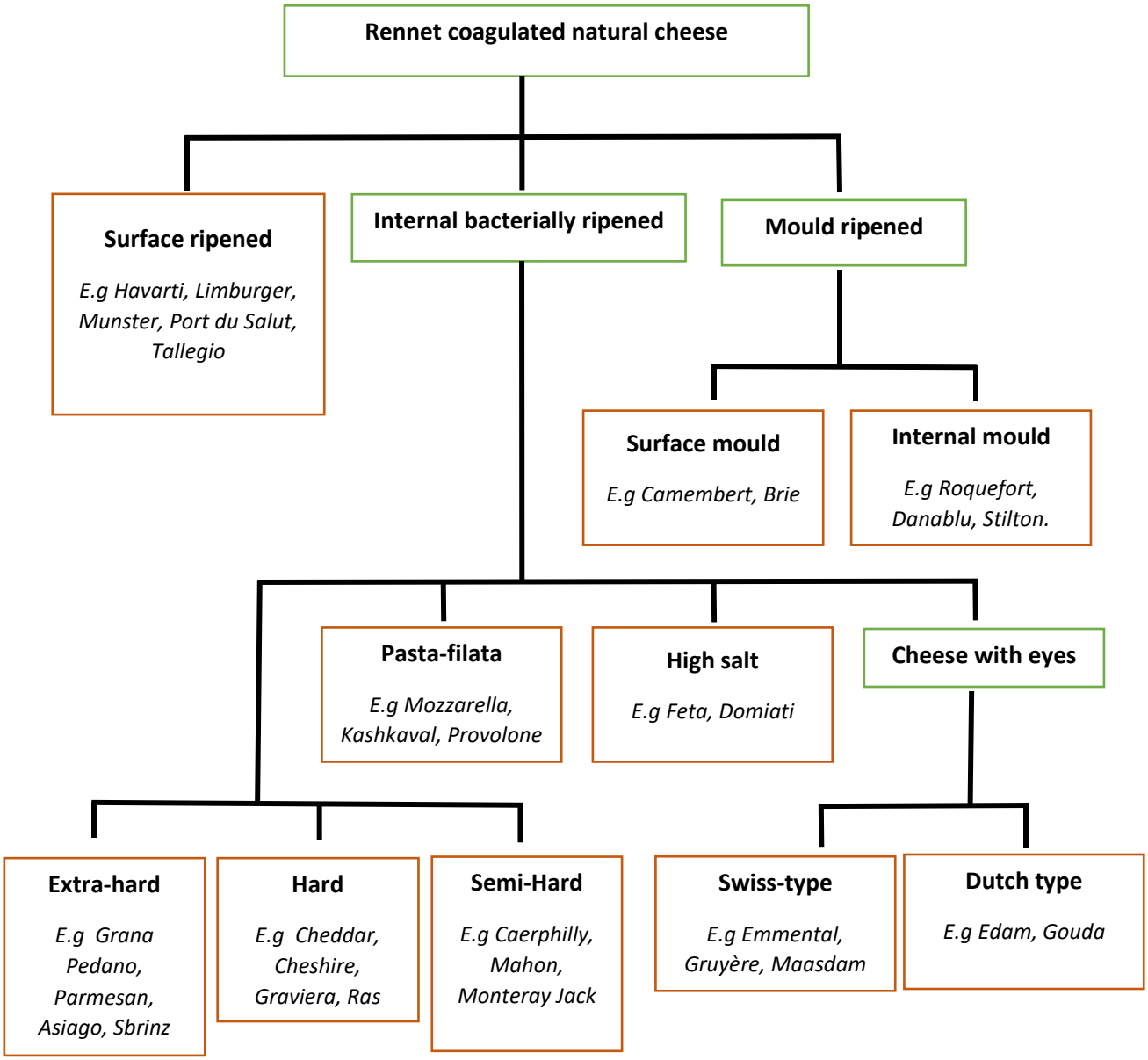
2.1 Cheese varieties

Cheese is the curd product of coagulated milk, after the whey has been drained. The curd is then shaped, and in many cases ripened. This broad definition is required to encompass the wide diversity of cheese varieties, as estimates suggest there may be as many as 1400 globally (McSweeney, 2007).

Cheeses can be categorised primarily by their method of achieving coagulation (rennet, acid, heat and acid) and then secondarily by other technological differences in their production. Cheesemakers control numerous variables during cheesemaking

that determine the texture, flavour and variety of cheese produced. These include the origin of the milk (cow, buffalo, sheep etc), the starter culture, use of rennet, additional cheesemaking steps (such as Cheddaring, pasta filata etc) and the ripening period (Fox & McSweeney, 1998).

Figure 2.1 Classification model for rennet coagulated cheeses.



Adapted from (McSweeney, 2007)

Figure 2.1 shows a classification structure for rennet coagulated cheeses, which make up 75% of cheese varieties (McSweeney, 2007). It classifies cheeses into surface

ripened, surface mould ripened, internal mould ripened, pasta-filata type, high salt, Dutch-style cheese with eyes, Swiss-style cheese with eyes, semi-hard, hard and extra-hard cheeses. Aside from these, there are further categories of acid-coagulated cheeses (e.g. cottage cheese, quark) and heat with acid coagulated cheeses (e.g. ricotta).

2.2 Cheesemaking and maturation

Milk is an emulsion of fat globules suspended in an aqueous solution comprised of proteins (predominantly casein), carbohydrates (predominantly lactose) and other minor components (Fox & McSweeney, 1998). The casein molecules in milk self-assemble into spherical nanostructures called micelles, in which the most hydrophilic region of the casein molecules is oriented on the outside of the sphere. (Dalgleish & Corredig, 2012).

Cheese is produced by coagulation of milk proteins, followed by separation of liquid whey from solid curds. Coagulation is initiated by acidification, often by the addition of starter cultures consisting of bacteria which convert lactose into lactic acid (Fox & McSweeney, 1998). Alternatively, or in addition to acidification, coagulation is induced by addition of rennet or rennet replacements such as chymosin, which contain enzymes that neutralise casein micelles and cause casein to aggregate, producing the solid known as curds. The remaining moisture and non-aggregated protein from the milk are retained in the whey, which is separated from the curds before they are pressed to achieve the desired shape and texture. Some cheeses go through additional processing steps, such as the pasta-filata process of curd stretching which produces the characteristic string-like structure in mozzarella.

Depending on the cheese variety, most cheeses are also matured before consumption. During maturation cheeses are stored at strictly controlled temperature and humidity to facilitate specific changes in flavour and structure. The maturation period varies from days to years depending on the cheese and desired maturity level. (Fox & McSweeney, 1998)

Table 2.1 Typical aging periods for cheese types explored in this thesis.

Cheese	Typical aging period
mozzarella	<1 month
mild Cheddar	3 months
mature Cheddar	9 months
Parmesan	2 years +

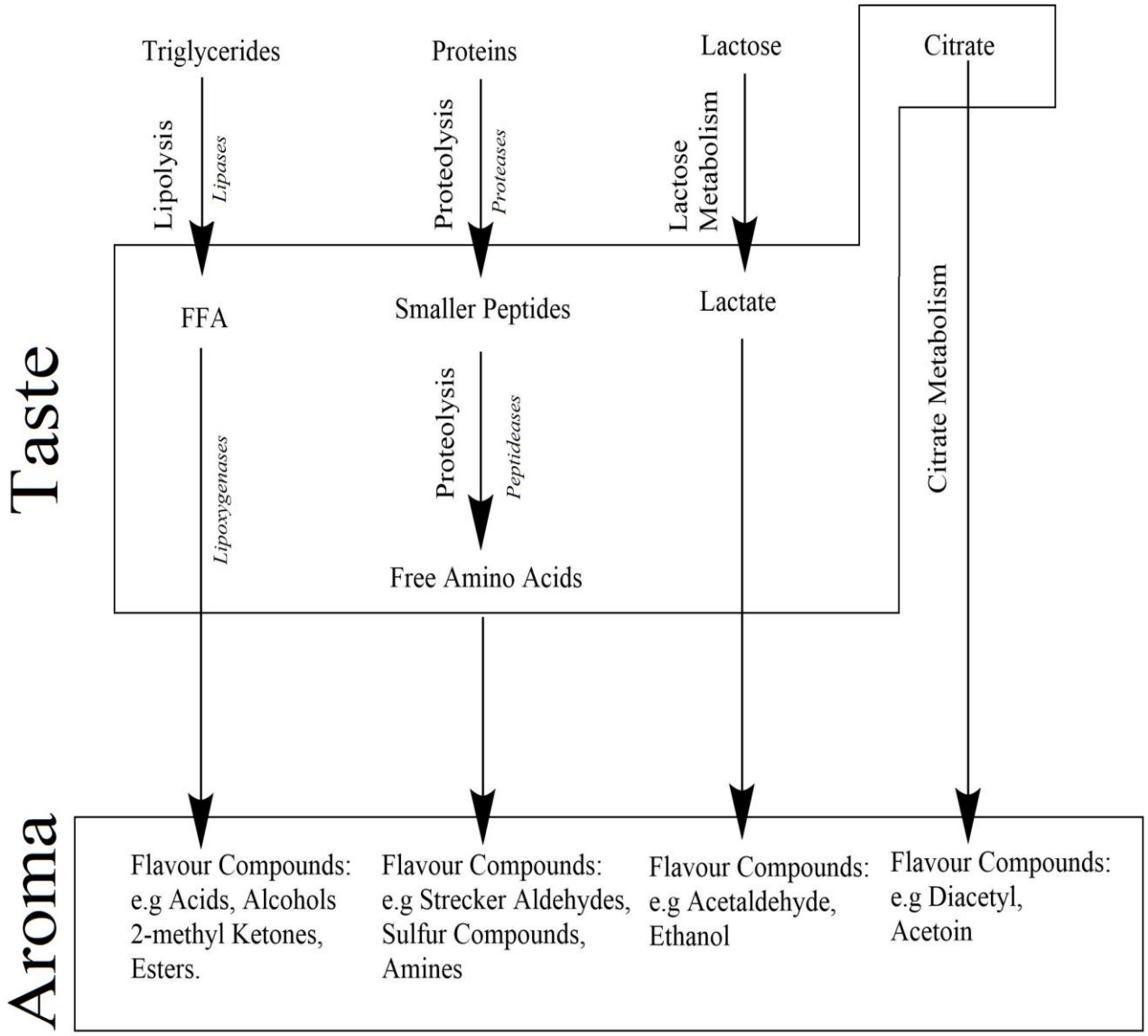
(McSweeney, 2011)

Cheddar is the most popular cheese in the UK (Kantar Media, 2021b). Cheddar is produced from cow's milk using starter culture and rennet. After Cheddar curds are cut, they are pressed into loaves and stacked on top of each other, reducing residual moisture and creating the characteristic crumbly texture of Cheddar, in a process known as Cheddaring. The curds are then milled, dry salted and pressed into moulds.

Cheddar is matured for a minimum of 2-3 months, at which point it is termed mild Cheddar, depending on the maturation length Cheddar can also be considered medium (6 months), mature (9 months), extra mature (15 months) or vintage (at least 18 months) (Singh et al., 2003a). In comparison to Cheddar, traditional mozzarella

538 is minimally or not aged and relatively high moisture, while Parmesan is an extra-
539 hard cheese which is extensively aged before consumption.

540 Figure 2.2 Overview of key processes impacting flavour development during
541 cheese ripening



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543 (McSweeney, 2011)

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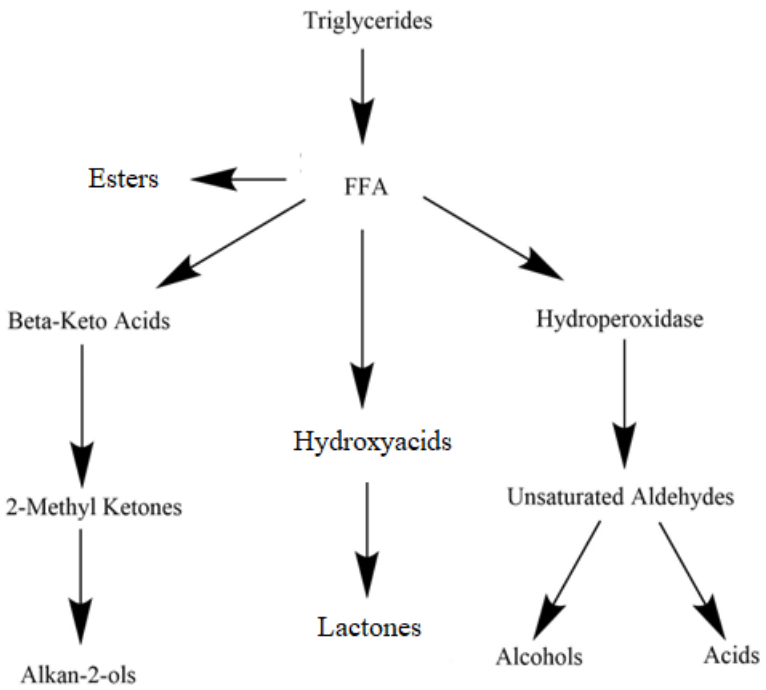
Chemical processes that occur during cheesemaking and ripening are responsible for developing the characteristic and varied flavours of cheeses from the starting milk. The majority of cheese flavour is formed during maturation through three processes: proteolysis, lipolysis and lactate and citrate metabolism (McSweeney, 2011).

The three processes are described below. The products generated during these processes participate in further reactions and contribute to formation of additional flavour compounds with a wide variety of functional groups in matured cheese.

2.2.1 Lipolysis

Figure 2.3 The lipolysis reaction cascade including key reactions of FFA in cheese.

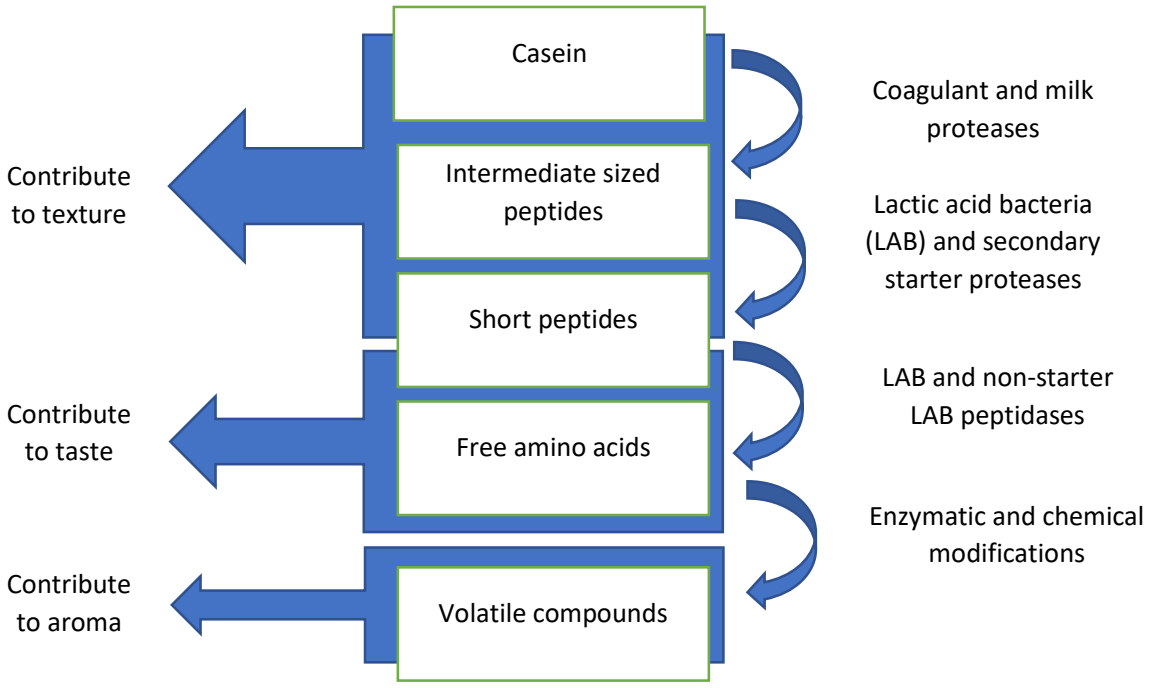


(McSweeney, 2011); (Collins et al., 2003)

Milk fat is comprised of triglycerides of fatty acids. Lipolysis is the hydrolysis of triglycerides into free fatty acids (FFA), glycerol and mono or diglycerides. The majority of lipolysis in cheese is performed by lipase enzymes, which originate both from the milk itself and the bacteria, yeasts and moulds which make up the cheese microflora (McSweeney, 2011; Collins et al., 2003). The enzymes facilitate hydrolysis of the ester linkages between fatty acid and glycerol moieties of triglycerides, generating FFA. Small and medium chain FFA are important cheese odorants and longer chain fatty acids contribute to fatty taste (Rychlik & Bosset, 2001a). FFA also act as precursors to other odorants such as esters, 2-methylketones, alcohols, aldehydes and lactones.

2.2.2 Proteolysis

Figure 2.4 The proteolysis reaction cascade.



Adapted from (Feijoo-Siota et al., 2014).

(Collins et al., 2003; McSweeney, 2011) Lipolysis is especially important in the development of flavour of mould-ripened cheeses, where secondary microflora develop which are strongly lipolytic (McSweeney, 2011); (Collins et al., 2003) .

Proteolysis is the hydrolysis of proteins by proteinases and peptidases into shorter peptides and individual amino acids, as outlined in figure 2.4. It is considered the most important process for development of cheese flavour in most varieties of cheese (McSweeney, 2011). The peptides and amino acids generated during proteolysis contribute to bitter, umami and kokumi tastes in cheese (Feijoo-Siota et al., 2014; McSweeney, 2011). Furthermore, they are precursors to the generation of various aroma compounds, including aldehydes, branched chain acids, alcohols, esters, sulfur compounds and amines. Proteolysis also contributes to the structural and textural changes which occur during the transformation between fresh curd and matured cheese (McSweeney, 2011). During proteolysis, the hydrolysis of casein into smaller peptides and amino acids reduces the strength of the casein network, resulting in a texture change from a springy/plastic-like texture to more crumbly and non-cohesive (Lawrence et al., 1987).

2.2.3 Lactose and citrate metabolism

Lactose is metabolised by *Lactococcus* bacteria in cheese to produce lactate within the first days and weeks of ripening (McSweeney, 2011). The mechanism of lactose metabolism involves cleavage of lactose to produce the monosaccharides glucose and galactose, followed by a series of bacteria driven reactions which ultimately produce lactate and ethanol. Lactate formation lowers the pH of the cheese, influencing which bacterial strains are most prevalent and the rate of generation of many other flavour compounds (McSweeney et al., 2000). Depending on the variety

of cheese, bacteria (introduced in the starter culture and encouraged to grow by suitable ripening conditions) may further metabolise lactate (Fox & McSweeney, 1998). For example, *Propionibacterium spp* in Swiss cheese metabolises lactate to form propanoic acid, acetic acid and carbon dioxide (Fox & McSweeney, 1998; McSweeney, 2011). The acids are thought to contribute to the flavour of Swiss cheese, while the carbon dioxide is responsible for the formation of holes in the cheese structure (Fox & McSweeney, 1998).

Citrate is found in cheese curds and can be metabolised by some strains of *lactococci* (McSweeney, 2011). Products of citrate metabolism include 3-hydroxy-2-butanone (acetoin), acetate, 2,3-butanedione and 2,3-butanediol (McSweeney, 2011). Pyruvate is also formed during citrate metabolism and can be reduced to lactate, such that further products of citrate metabolism also include the products of lactate metabolism discussed above (Liu, 2003).

Lipolysis, proteolysis and lactose and citrate metabolism collectively contribute towards the primary metabolism of flavour precursors in cheese during ripening. These processes affect the structure, rheology, taste and aroma properties of the cheese. While some of the important odorants are formed directly during this primary metabolism, many more are formed during secondary processes which act on precursors formed during the primary phase. It is generally accepted that the flavour of cheese depends on the combination and balance of many components, rather than on any one key odorant. This theory is known as ’component balance theory’ (Bosset & Gauch, 1993; Mulder, 1952).

2.3 Characterisation of cheese

2.3.1 Cheese structure

The structure of cheese is an amorphous network of interlinked caseins held together by divalent calcium ions and hydrophobic interactions, interspersed with water and globules of fat (Everett & Auty, 2008).

Factors influencing cheese structure include:

a. Maturation (see section 2.2)

b. Composition of the cheese

Interspersed fat and water disrupt the casein network, lessening the number of interactions between casein molecules (Mistry, 2001). High moisture content is associated with softness in cheese, and reduced fat content is associated with high protein aggregation and a firmer, more rubbery texture (Ardö, 1997; Guinee et al., 2000; Mistry, 2001; Tunick et al., 1993; Vítová et al., 2007). Salt content, protein content and calcium content are other important compositional factors (Everett & Auty, 2008).

c. Cheese pH.

Lower pHs generate cheeses with a more crumbly, less cohesive texture (e.g. Cheshire, feta) (Lawrence et al., 1987). In uncooked cheese the structure is therefore a balance between the strength of the casein network, which is influenced by the pH and alters during ripening, and the levels of moisture and fat.

Significant structural changes occur during cooking of cheese. During heating, the fat globules melt enabling the cheese to flow. Hydrophobic interactions between

casein molecules maintain an element of solid structure which allows melted cheese to stretch to a point without breaking. Mozzarella, especially, has a highly developed casein structure due to the pasta filata process, which enables heated mozzarella to stretch considerably when pulled apart (Everett & Auty, 2008). However, once the fat begins to coalesce into pools or a layer, it no longer interrupts the casein structure, allowing for higher levels of hydrophobic casein interactions to develop. Extensively cooked cheese is therefore firm and less prone to stretch (Fox et al., 2016; Kuo et al., 2001; Wang & Sun, 2003).

Low-fat cheeses are less susceptible to melting and stretching upon heating than their full-fat counterparts (Guinee et al., 2000; Guinee & Kilcawley, 2004; Mistry, 2001; Tunick et al., 1993; Vítová et al., 2007). The reduced level of fat and stronger casein structure in low-fat cheeses restricts the ability of heated low-fat cheese to flow.

2.3.1.1 Methods for characterisation of cheese structure

Characterisation of microstructure is performed using microscopy techniques, which enable visualisation of the structure of cheese down to the nanometre scale. Two microscopy techniques that have been widely used on cheese are scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) (Everett & Auty, 2008).

SEM generates a visualisation of the surface topology of the specimen, by scanning the surface with a beam of electrons and measuring secondary electrons that are ejected from the sample. SEM has been used to visualise the size, shape and distribution of different phases within cheese, including protein structure, fat globules and pores (El-Bakry & Sheehan, 2014). During CLSM, laser scanning generates 2D images which are computationally combined to generate a 3D model

of the cheese structure (El-Bakry & Sheehan, 2014). Microscopy techniques have been used to study the structure of cheeses with low and high fat and structural changes during cooking, such as coalescence of fat globules (Bryant et al., 1995; Paquet, 1988).

2.3.2 Cheese taste

The taste of foods is perceived by receptors on the tongue and is a combination of the basic tastes; sweet, bitter, salty, sour, umami (savoury taste) and kokumi (deliciousness, enhancing taste). In cheese, mineral salts, lactic acid, sugars, amino acids and low molecular weight peptides are all potentially taste active (Engel et al., 2000, 2001; Warmke et al., 1996).

The basic tastes are susceptible to taste interactions with one another, which can be either enhancing, suppressing, synergistic or masking (Breslin, 2001). For example, sour taste in cheese is reported to suppress sweetness, such that cheeses perceived as sweet are often characterized by having low concentrations of sour tastants, rather than necessarily high concentrations of sweet tastants (McSweeney, 1997; Niimi et al., 2014).

2.3.2.1 Sweetness, saltiness and acidity in cheese

Saltiness is one of the predominant tastes in cheese and considered a positive attribute. Sodium chloride (NaCl) is added during cheesemaking, originally to preserve the cheese and to control water activity (McSweeney, 1997). NaCl is the most important contributor to saltiness in cheeses (Hillmann & Hofmann, 2016), but other salts including potassium chloride, calcium chloride and magnesium chloride also contribute (Engel et al., 2000; 2001).

Sourness is also integral to the flavour of most cheeses, especially mature cheeses, although high levels of sour flavour can be considered a flavour defect (Smit et al., 2009). Lactic acid is one of the main sources of sour taste in cheese (Hassan et al., 2013). As a product of lactose metabolism, lactic acid concentration and cheese sourness increase during cheesemaking and the early stages of maturation. Other acids, such as acetic, butanoic and propanoic acid are formed during maturation and contribute to the aroma of cheese along with sour taste, although their contribution to taste is less important than that of lactic acid (Hassan et al., 2013). Sourness in cheese depends on variety, pH and on the concentration of other taste compounds such as NaCl. (McSweeney, 1997).

Sweetness is an important character of some cheese flavour, although less important in most cases than other tastes such as saltiness and acidity. As lactose is a key contributor of sweet flavour in milk (Nursten, 1997), the presence of lactose, galactose and glucose is also associated with sweetness in curd or young cheese. As lactose is metabolised during maturation, cheeses with lengthy maturation periods have low concentrations of milk sugars. In mature cheeses sweetness is more likely to be related to products of proteolysis such as proline, or to the presence of calcium or magnesium ions than to lactose and galactose (McSweeney, 1997; Kilcawley, 2017)

2.3.2.2 Bitterness in cheese

As with sour taste, some bitterness is characteristic in cheese, while excessive bitterness is considered a flavour defect (Smit et al., 2009). Bitterness in uncooked cheese is linked to the presence of certain salts (e.g. calcium chloride), amino acids (e.g. leucine, isoleucine) and peptides (Hillmann & Hofmann, 2016; McSweeney,

1997; McSweeney, 2007). Of these groups, the peptides are believed to be important, but their characterisation has proved most complex due to the large numbers of small chain peptides produced by proteolysis.

Taste omission experiments have determined that peptide fractions of molecular weight 400 – 3000 Da are key contributors for bitter taste, corresponding to short chain peptides ranging from two to twenty amino acids in length. (Engel et al., 2001; Lemieux & Simard, 1992; McSweeney, 2007; Toelstede & Hofmann, 2008). The specific amino acids and chain length of bitter peptides varies, but the degree of bitterness is related to the mean hydrophobicity of the amino acid chains, along with the nature of the amino acids at the peptide terminals and the steric parameters of the chain (Lemieux & Simard, 1992) . There is not yet a conclusive list of peptides responsible for bitterness in cheese. Factors including cheese variety, cheesemaking cultures and length of maturation period are all likely to affect the peptides produced. In particular, the fragment β-casein (CN) (193-209) and its subfragments β CN (198-206) and β CN (198-208) have been reported in multiple studies (Karametsi et al., 2014; Singh et al., 2005; Toelstede & Hofmann, 2008).

In addition to linear peptides, diketopiperazines (DKPs) are cyclic dipeptides which contribute to bitter taste in many cooked foods (e.g. beef (Chen et al., 2009), bread (Ryan et al., 2009), coffee (Ginz & Engelhardt, 2000)). They are formed either enzymatically during fermentation, or during thermal processing (Borthwick & da Costa, 2017; Chen et al., 2009; Ginz & Engelhardt, 2000; Ryan et al., 2009). While DKPs (cyclo-Pro-Pro, cyclo-Pro-Val, cyclo-Pro-Phe, cyclo-Pro-Leu, cyclo-Pro-Ala) have previously been reported in uncooked cheese, their presence has been shown to be sub-threshold and therefore they are not thought to be important contributors to bitterness in uncooked cheese (Roudot-Algaron et al., 1993.). However, the

prevalence of DKPs generally in cooked foods suggests that the presence and concentration of DKPs in cooked cheese warrants further exploration.

2.3.2.3 Umami and kokumi in cheese

Umami and kokumi are important contributors to the taste of some uncooked cheese, especially mature cheeses (Hillmann & Hofmann, 2016; Zhao et al., 2016). Like bitterness, free amino acids and peptides are associated with umami and kokumi taste in cheese. Glutamic acid is a key functional group which is common to peptides with umami and kokumi character. Glutamate and some α-glutamyl peptides contribute to umami taste in uncooked cheese, while γ-glutamyl peptides exhibit kokumi taste (Drake et al., 2007; Zhao et al., 2016). Both α and γ peptides are formed in cheese during proteolysis, forming from casein and the action of γ-glutamyl transferase on amino acids respectively (Tunick et al., 1993; Zhao et al., 2016; Kilcawley, 2017).

2.3.3 Methods for characterising non-volatiles in cheese

Proximate analysis is used to determine approximate levels of food components such as fat, protein, and moisture using experimental techniques. Proximate analysis is routinely used on cheese as an inexpensive way to approximate the levels of such components and to compare cheese samples.

The analysis of non-volatile components such as peptides, individual amino acids and sugars is commonly achieved through liquid chromatographic techniques. The process for identifying tastants in cheese involves the extraction of water-soluble components in a water-soluble extract (WSE) (Toelstede & Hofmann, 2008), followed by fractionation by molecular size and tasting of the fractions to identify the taste properties they possess. Individual compounds within fractions are identified using liquid chromatographic methods, by comparison against authentic

standards. The addition of mass spectrometry in LC-MS enables determination of the molecular weights of the analytes, which can aid in confirming identification. In the case of complex molecules, such as peptides, compositional and structural determination is performed using liquid chromatography tandem mass spectrometry (LC-MS-MS). As LC-MS is only able to determine the molecular mass of a molecule, it is not suitable for determining the amino acid sequences of peptides with three or more amino acids. LC-MS-MS can separate specific fragments of the molecule and perform further fragmentation on them. this technique enables differentiation between structural isomers (Karametsi et al., 2014; Singh et al., 2005; Toelstede & Hofmann, 2008).

Once tastants are identified, they are synthesized and tasted to confirm their taste character. However, in some cases elements from the matrix interact with tastants, enhancing or reducing the intensity of their taste. To account for this effect, taste omission experiments are often performed. The composition of a WSE is recreated as accurately as possible in a model mixture, which is tasted against versions of the model with specific molecules omitted to fully characterise their effect in the mixture (Karametsi et al., 2014; Singh et al., 2005; Toelstede & Hofmann, 2008).

2.3.4 Cheese aroma

The range of volatile components which contribute to the flavour of uncooked cheese are various and depend on cheese type and processing conditions. Odorants identified in uncooked cheese using GC-O are outlined by functional group in sections 2.3.4.1 to 2.3.4.12.

2.3.4.1 Fatty Acids

Fatty acids are carboxylic acids with straight or branched aliphatic chains. Short chain fatty acids such as butanoic and hexanoic acid, are common odorants in cheese and contribute to acidic, sweaty and cheesy flavour (Christensen & Reineccius, 1995; Drake et al., 2010; Inagaki et al., 2015.; Milo & Reineccius, 1997; Rychlik & Bosset, 2001b; Suriyaphan et al., 2001; Zehentbauer & Reineccius, 2002). Medium to long chain fatty acids impart waxy flavour in some cheeses, examples include decanoic, dodecanoic and tetradecanoic acid (Collins et al., 2003).

Most of the fatty acids in cheese are bound in triglycerides originating from milk fat, some of which are released during lipolysis (Collins et al., 2003; McSweeney, 2011). In most cases the ratio of FFA in cheese is similar to the ratio in milk, except for butanoic acid, which is found at a higher proportion relative to the other acids. Butanoic acid is preferentially cleaved by starter esterases due to its triglyceride backbone position (Wilkinson et al, 2011).

Fatty acids are susceptible to a range of further reactions and act as precursors to many other odorants in cheese, including 2-methylketones, secondary alcohols, aldehydes, lactones and esters.

2.3.4.2 Ketones

As part of lipolysis, certain cheese moulds (e.g. Penicillium roqueforti) decarboxylate fatty acids producing 2-methylketones with one fewer carbon in their chains (Collins et al., 2003). As even carbon chain numbered fatty acids are the most prevalent in cheese (e.g. butanoic acid, hexanoic acid), the most prevalent 2-methylketones are odd carbon chain numbered. Ketones commonly reported in mould-ripened cheeses include 2-heptanone, 2-nonanone and 2-undecanone, all of which have characteristic blue cheese aromas. (Poveda et al., 2008; Piombino &

Addeo, 2000; Preininger & Grosch, 1994; Qian & Reineccius, 2003a, 2003b). Fatty acids aren't the only precursors to methylketone formation, small quantities of ketoacids present in milk fat may also be transformed by mould in cheese into methylketones (Collins et al., 2003).

1-Octen-3-one is another important ketone previously reported in Cheddar, camembert and goats' cheese (Carunchia Whetstine et al., 2003; Kubeckova & Grosch, 1997; Suriyaphan et al., 2001; Zehentbauer & Reineccius, 2002). It has a strong earthy aroma and is thought to form from products of lipolysis, specifically the breakdown of fatty acids such as linoleic acid (Ray, 2017). Other ketone odorants in cheese include 2,3-butanedione and 3-hydroxy-2-butanone (Christensen & Reineccius, 1995; Poveda et al., 2008). These buttery components are formed from citrate and lactose metabolism (McSweeney, 2011).

2.3.4.3 Alcohols

A small number of alcohols have been found to be important odorants in cheese, predominantly in mould ripened cheeses such as blue cheese, brie and camembert. Formation of alcohols in cheese occurs via the reduction of corresponding carbonyl compounds (McSweeney et al., 2000). 2-Heptanol is an example of an alkan-2-ol, many of which are formed in cheese through reduction of 2-methylketones. 2-Heptanol has a fruity aroma and is a product of 2-heptanone. Similarly, 1-octen-3-ol is formed from reduction of 1-octen-3-one, and has a potent earthy aroma (Kubeckova & Grosch, 1997; Piombino & Addeo, 2000; Qian & Reineccius, 2003b, 2003a).

The similar reduction of aldehydes yields primary alcohols, for example 3-methylbutanol has a fruity aroma and is formed from reduction of 3-methylbutanal, a product of the Ehrlich pathway (see figure 2.5) (Ehrlich, 1907).

2.3.4.4 Esters

The most important and widely reported class of esters in cheese are ethyl esters, especially ethyl butanoate and ethyl hexanoate (Avsar et al., 2004; Inagaki et al., 2015.; Kubeckova & Grosch, 1997; Piombino & Addeo, 2000; Qian & Reineccius, 2003b, 2003a; Suriyaphan et al., 2001; Zehentbauer & Reineccius, 2002). Ethyl esters are a product of esterification reactions between ethanol (formed by fermenting lactose and usually the rate limiting reagent) and FFA (from lipolysis) catalysed by enzymes (Smit et al., 2009; McSweeney, 2011). Alternatively, formation of esters has been shown to occur via alcoholysis (the reaction between an alcohol and an ester, forming another ester) and an acylglycerol or acyl-CoA derivatives (Liu et al., 2004). Esters impart a fruity note to cheese, which can be considered an off note in samples with very high ester content (McGugan et al., 1975).

2.3.4.5 Lactones

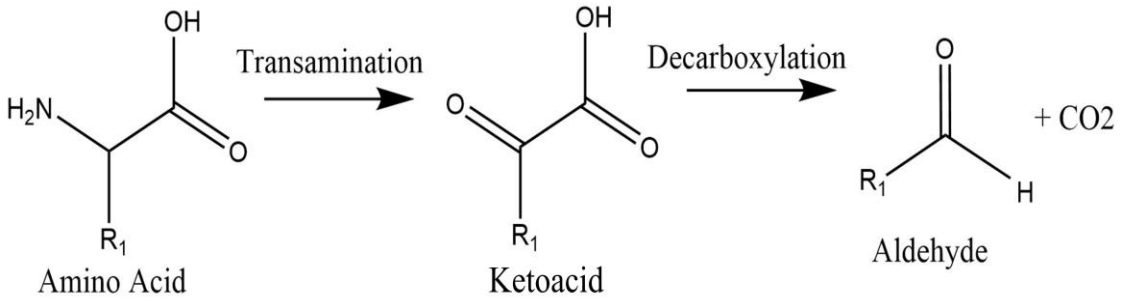
Lactones are cyclic esters that impart creamy aromas (Avsar et al., 2004; Inagaki et al., 2015.; Kubeckova & Grosch, 1997; Poveda et al., 2008). Lactones are lipid-derived, formed via trans-esterification of hydroxy fatty acids in their triglyceride form Collins et al., 2003; Kishimoto et al., 2003). As lactone formation is faster at increased temperature, lactones may be expected to be important to the volatile profiles of cooked cheese (Alewijn et al., 2007).

2.3.4.6 Aldehydes

Aldehydes found in cheese fall generally into two groups: those formed by lipid oxidation and those formed during the degradation of amino acids. Lipid oxidation generates saturated aldehydes, such as hexanal, unsaturated aldehydes such as trans-2-nonenal and dienals such as 2,4 decadienal. These lipid-derived aldehydes typically have fatty or green aromas and are relatively uncommon in cheeses with the exception of hexanal. (Collins et al., 2003).

Catabolism of amino acids into aldehydes in cheese occurs via the Ehrlich pathway catalyzed by enzymes (Ehrlich, 1907). Common products of the Ehrlich pathway cheese include 2 and 3-methylbutanal (malt like aroma), 2-methylpropanal (malt like aroma) and phenylacetaldehyde (floral), which are formed from isoleucine, leucine and phenylalanine respectively (Kubeckova & Grosch, 1997; Piombino & Addeo, 2000; Preininger & Grosch, 1994; Zehentbauer & Reineccius, 2002).

Figure 2.5 Overview of the Ehrlich Pathway



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(Ehrlich, 1907)

2.3.4.7 Sulfur compounds

Volatiles containing one or more sulfur atoms often have strong aromas. 3-Methylsulfanylpropanal (methional) is a sulfur-containing aldehyde formed via the

Ehrlich pathway from methionine (Avsar et al., 2004; Inagaki et al., 2015.; Poveda et al., 2008; Suriyaphan et al., 2001). Furthermore, enzymatic transformations in cheese convert methionine to methanethiol, which oxidises to other sulfides. (Smit et al., 2009). In particular, dimethyl trisulfide is a key odorant in cheese with an alliaceous aroma. (Kubeckova & Grosch, 1997; Qian & Reineccius, 2003b, 2003a; Suriyaphan et al., 2001; Zehentbauer & Reineccius, 2002)

2.3.4.8 Furanones and Pyranones

Furanones are heterocyclic molecules, often with cooked aromas. Of the furanones, 4-hydroxy-2,5-dimethylfuran-3(2H)-one (furaneol) is most widely reported as an odorant in cheese (Drake et al., 2010; Inagaki et al., 2015.; Milo & Reineccius, 1997; Qian & Reineccius, 2003a; Suriyaphan et al., 2001; Zehentbauer & Reineccius, 2002). It is commonly found in Cheddar along with 2-ethyl-4-hydroxy-5-methylfuran-3(2H)-one (homofuraneol). Furaneol and homofuraneol have caramellic odours and are thought to be formed by certain strains of lactic acid bacteria (Milo & Reineccius, 1997) or by Maillard reactions between sugars and amino acids.

2.3.4.9 Pyrazines

Pyrazines are aromatic six membered rings containing two nitrogen atoms in the 1 and 4 ring positions respectively. They have been shown to contribute to nutty notes in cheese (Qian & Reineccius, 2003a). Pyrazine formation is thought to occur through reactions between carbonyl compounds and amino acids. For example, Griffith and Hammond demonstrated that 2,5 dimethylpyrazine formed from the reaction of ornithine and dihydroxyacetone under similar pH and water activities to those found in cheese. (Griffith & Hammond, 1989)

In some cases, pyrazines have been shown to contribute to aroma defects. For example, the bell pepper note detected in British Farmhouse Cheddar by Suriyanphan et al (Suriyaphan et al., 2001) was attributed to 2-isobutyl-3-methoxypyrazine. Methoxy pyrazines form from cheese microflora rather than in the Maillard reaction (Neta et al., 2008).

2.3.4.10 Other nitrogen-containing heterocyclic compounds

Aside from pyrazines, other nitrogen-containing heterocyclic compounds possess widely varying aroma characteristics and are formed from amino acid precursors (Yokoyama & Carlson, 1979). Examples in cheese include indole and 3-methylindole (skatole) which are both animalic in aroma, while 2 acetyl-1-pyrroline has an aroma reminiscent of popcorn (Avsar et al., 2004; Drake et al., 2010; Preininger & Grosch, 1994; Zehentbauer & Reineccius, 2002).

2.3.4.11 Terpenes

Terpenes are found in milk and cheese from cows which consumed a terpene-rich diet, for example high mountain pastures (Noni & Battelli, 2008). They are thought to transfer directly from the bovine food source into the milk, rather than forming during cheesemaking or maturation. While terpenes have been reported in cheese, they are not thought to be major odorants.

2.3.4.12 Phenolics

Phenolic compounds possess woody, smoky or faecal aromas and are considered to contribute to defect flavour in cheese. Their formation pathways are not clear, but phenolic compounds may form from lactic acid bacteria in cheese, as has been

demonstrated for the formation of 4-methylphenol (p-cresol) in Gouda (Badings et al., 1968). Phenolic compounds are also abundant in smoked cheese, where they originate from the smoking process (Palencia et al., 2014).

2.3.5 Characterisation techniques for odour-active volatiles

Identification of odorants in foods is typically performed using gas chromatography mass spectrometry (GC-MS) in conjunction with gas chromatography-olfactometry (GC-O) for the separation and identification of volatile compounds and their respective aromas (Elmore, 2015b; van Ruth, 2001).

Before GC analysis, volatile compounds are isolated from the non-volatile food matrix by various extraction techniques. Choice of extraction technique can have a significant effect on the results of the analysis. Extraction techniques are broadly split into two categories, solvent and headspace extraction, both of which have been applied to cheese. (Arora et al., 1995; Bertrand et al., 2011; Delgado et al., 2010; Frank et al., 2004; Lecanu et al., 2002; Mondello et al., 2005; Sánchez-Macías et al., 2011; Wang & Sun, 2002; Kilcawley, 2017).

2.3.5.1 Solvent Extraction

Solvent extraction techniques are commonly used in volatile analysis, as liquid extracts can be easily re-analysed multiple times for example to enable GC-O. Typical solvents include diethyl ether, dichloromethane or pentane; choice of extraction solvent affects the selectivity of the extraction and so the recovery of the odorants. Extraction efficiency may be limited by saturation of the solvent. To increase the extent of extraction, so-called exhaustive extraction using multiple portions of solvent has been employed (Avsar et al., 2004; Milo & Reineccius, 1997).

Alternatively, the Soxhlet apparatus (Soxhlet, 1879) enables continuous cycling of clean solvent through the sample matrix. In this way, the aroma is more effectively stripped than from a single solvent wash, without the use of additional extraction solvent. (Spinnler & Gripon, 2004).

Fat is often co-extracted from fatty foods along with volatile compounds, as many important odorants are hydrophobic and require lipophilic extraction solvents. For this reason, further techniques to remove fat from solvent extracts are required. Vacuum sublimation, or vacuum distillation was often employed for this purpose in literature pre-dating 2000. Around 2000, solvent assisted flavour evaporation (SAFE) replaced vacuum distillation as a more compact, easy to use technique (Engel et al., 1999). Both techniques employ a vacuum to separate volatile compounds, which evaporate upon entering the SAFE system, from non-volatile material which does not evaporate. A vessel immersed in liquid nitrogen is used to condense the volatiles.

Simultaneous dilution extraction (SDE) is an alternative extraction technique which has been employed for the study of cheese (Larráyoz et al., 2001; Singh et al., 2003b). During SDE, two flasks containing the sample dispersed in water and the extraction solvent respectively are heated to boiling. The vapours from the two flasks meet in a central condenser, where the vapours condense, and the volatiles transfer between sample vapour and solvent vapour. The condensed liquids flow back into their respective vessels. In this way, the solvent and fat from the sample never meet, and so subsequent fat removal is not necessary. However, as cheese is subject to thermal changes, use of SDE is prone to artefact formation. (Elmore, 2015a).

2.3.5.2 Headspace Extraction

During headspace extraction, volatiles are captured from the air around the sample (the headspace). Headspace techniques can complement liquid extractions (Larráyoz et al., 2001); as components which are poorly extracted during liquid extraction (due to losses during concentration or low solubility in the extraction solvent) may be detected in the headspace, however headspace techniques may struggle to detect higher boiling point volatiles.

Examples of headspace techniques which have been used on cheese include HS-SPME (Delgado et al., 2010; Frank et al., 2004; Lecanu et al., 2002; Mondello et al., 2005; Wang & Sun, 2002) and DHS (Arora et al., 1995; Bertrand et al., 2011; Sánchez-Macías et al., 2011; Vitova et al, 2007). HS-SPME involves capture and concentration of volatiles in the pores of the fibre (Elmore, 2015a). An equilibrium exists between volatiles in the food, in the headspace of the vial and on the fibre. This equilibrium and the limited volume of the HS-SPME fibre leads to competition between different compounds. This competition in the fibre and matrix effects from matrices such as cheese both cause challenges for making quantitative comparisons between samples using HS-SPME (Rincón et al, 2014).

During Dynamic headspace extraction (DHS), the sensitivity may be improved compared to HS-SPME by concentration of the headspace onto a volatile trap filled with a sorbent (e.g. Tenax TA). However, when extracting moist samples, it is necessary to purge water from the sorbent traps prior to GC analysis, which can lead to losses of the most volatile components. Additionally, to automate DHS sampling requires specific instrumentation as part of the GC-autosampler.

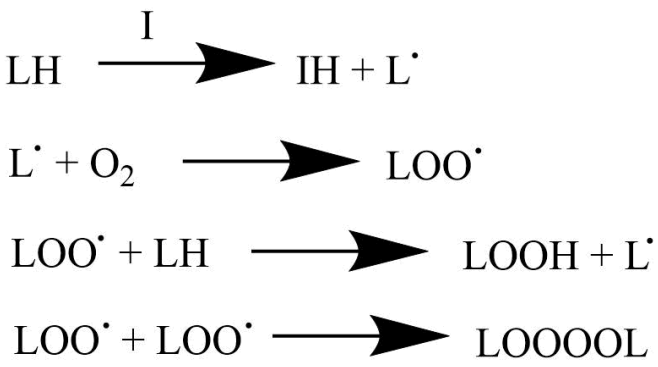
2.4 Thermally induced reactions

Changes in appearance, texture and flavour occur during cooking of all foods. The most important reactions which cause these changes are lipid degradation and the Maillard reaction. There is very little literature to date which addresses the changes in flavour when cheese is cooked, so this section will outline typical reactions which occur during cooking.

2.4.1 Lipid degradation

Lipid derived odorants include saturated, mono-unsaturated and di-unsaturated aldehydes which typically possess fatty, green or rancid aromas. They are formed from fatty acids by oxidation. Fatty acids are prone to autoxidation when exposed to oxygen, both during storage and, at an accelerated rate, during thermal processing (Frankel, 1998; Kanner & Rosenthal, 1992). When oxidation is thermally induced, as during cooking, the increased temperature provides additional activation energy which alters the selectivity for oxidation reaction pathways. This may explain some of the differences between lipids formed during oxidation at room temperature and during heating (Frankel, 1998; Kanner & Rosenthal, 1992).

Figure 2.6 Overview of the autoxidation of lipids.



Lipid oxidation occurs via a radical oxidation mechanism, as shown in figure 2.6. The lipid species becomes a radical after interaction with an initiator, I, (line 1) and forms a hydroperoxide by reaction with molecular oxygen (Line 2). Propagation (Lines 2-3) continues until two radical species react together, producing a non-radical product in the termination step (Line 4) (Frankel, 1998).As one mono-unsaturated lipid can have four different hydroperoxides, which can each cleave in one of two ways, the products of lipid oxidation are numerous (Frankel, 1998). Lipid oxidation products also react further with products from the Maillard reaction (Mottram, 1998).

2.4.2 The Maillard Reaction

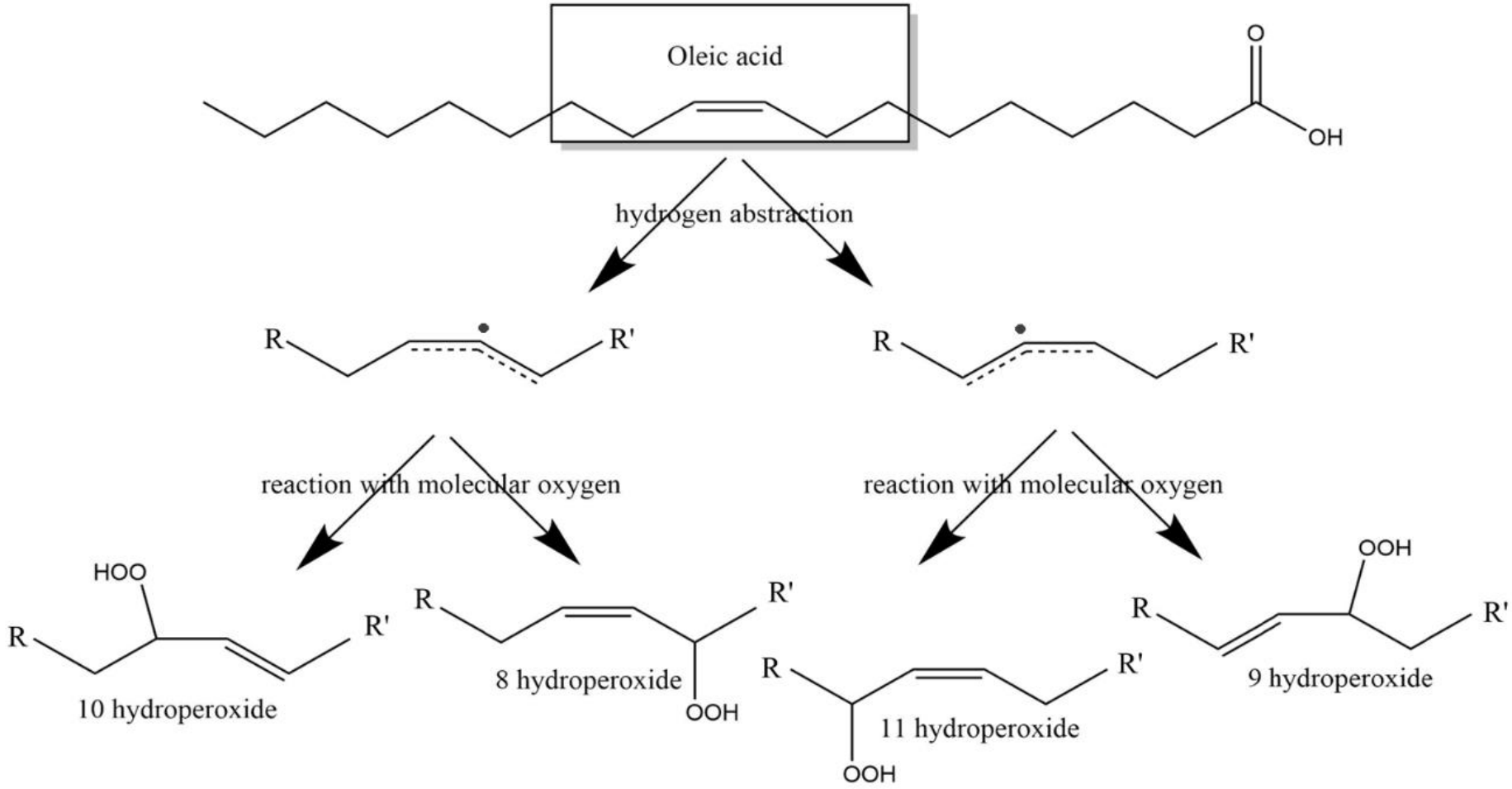
The Maillard reaction is a complex cascade of chemical transformations originating from the combination of an amino compound (e.g. protein) and a reducing sugar (Nursten, 1981). The Maillard reaction occurs slowly at room temperature, however its progress is rapid in the presence of heat. Maillard reaction products in real food systems are numerous (Nursten, 1981). They can be categorised as:

1. Products of the dehydration and fragmentation of the sugar moiety of the Amadori compound,
2. Degradation products of amino acids (e.g Strecker degradation products),
3. Volatiles formed from further interactions of products of the first two groups.

2.4.2.1 Early and Intermediate Stages of the Maillard Reaction

As shown in figure 2.8, an amino group and reducing sugar combine in the first stage of the Maillard reaction to form the N-substituted glycosylamine. This unstable

1024 Figure 2.7 Example of hydroperoxide formation from Oleic acid



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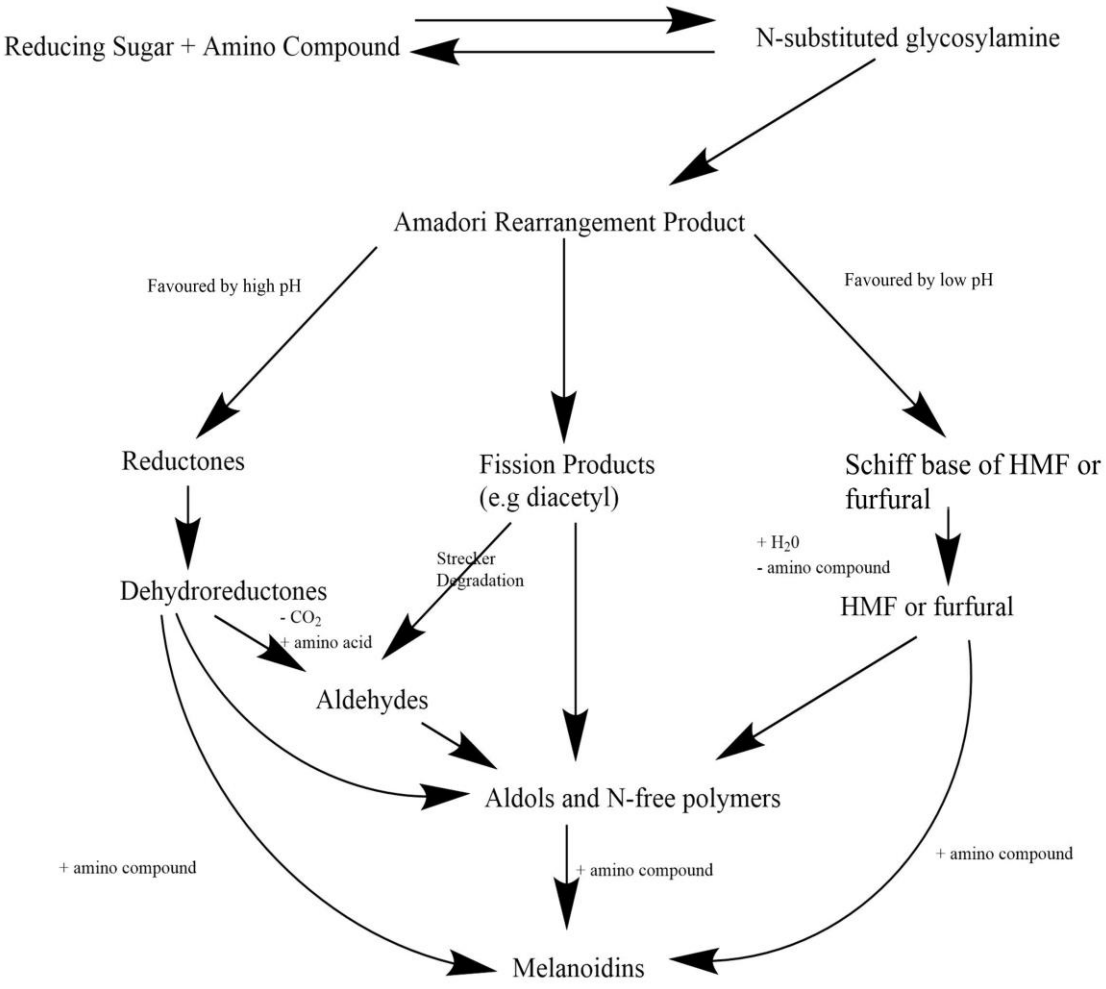
product readily rearranges to the Amadori product, which progresses along several different pathways, the relative likelihood of each is driven by reaction conditions such as the pH of the system, water activity and temperature. (Nursten, 1981)

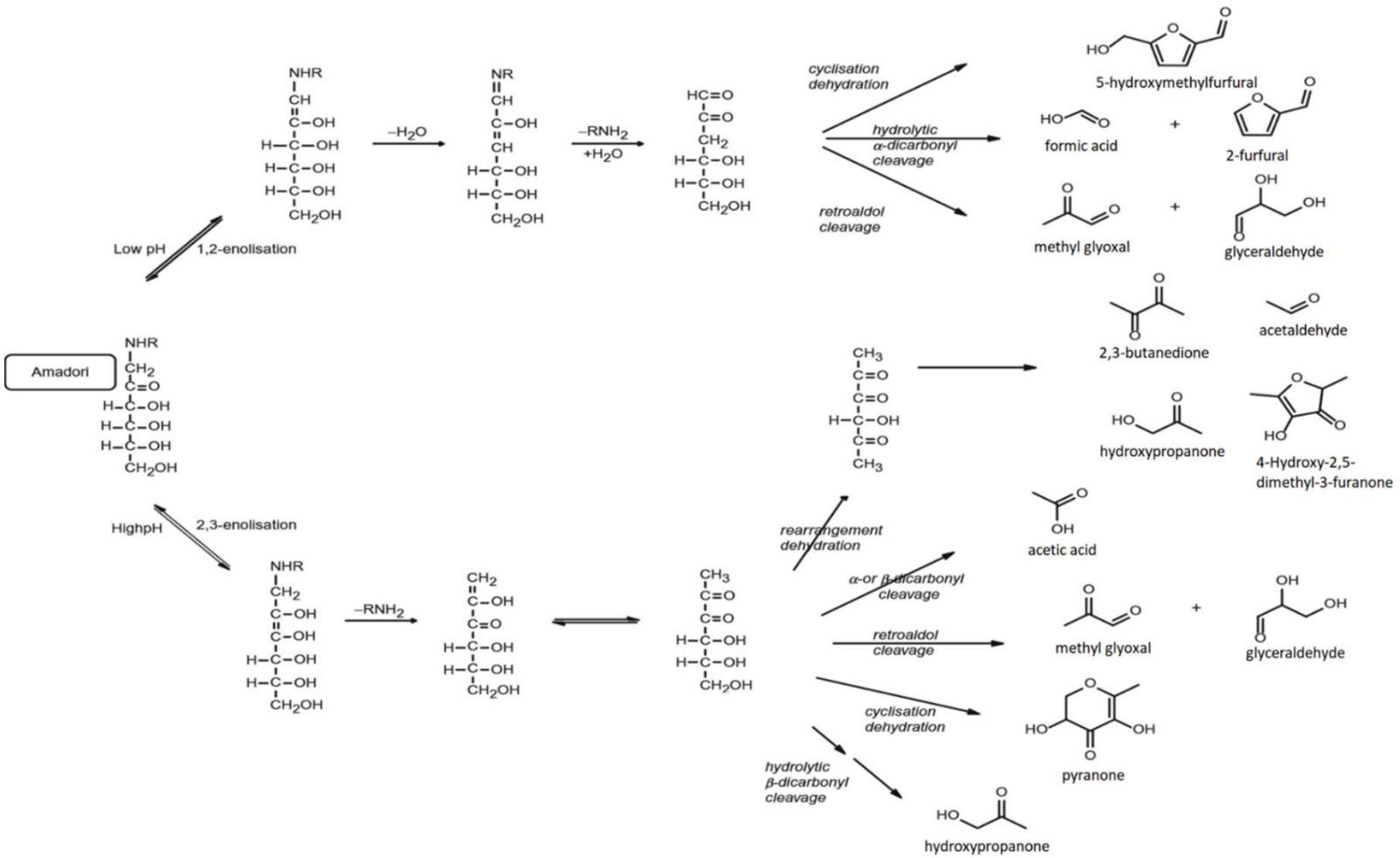
During retro-aldolisation, the bonds between the carbonyl group and the alcohol group are cleaved to give an enol and an aldehyde. The products of retro-aldol cleavage are small molecules (e.g acetic acid, 2,3-butanedione) which can be odorants in their own right or go on to react further.

Figure 2.8 Representation of the Maillard reaction as devised by Hodge.



(Hodge, 1953). HMF refers to hydroxymethylfurfural.

Figure 2.9 Representation of the intermediate stages of the Maillard reaction.



Adapted from Parker (2014).

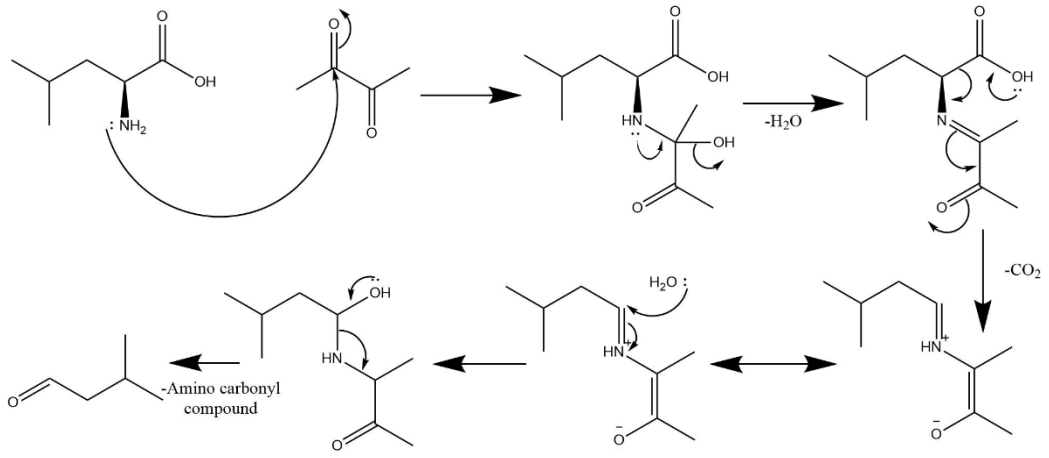
Reductone formation is favoured at higher pH and low moisture systems. Reductones are enediols, derived by the loss of two water molecules from a sugar. These reductones are precursors to furanones, pyrones and cyclopentenes. The retro-aldol products of sugar degradation are subject to react together once more in further aldol reactions which also lead to a range of similar cyclic products (Hodge, 1953).

Many of the products highlighted in figure 2.9 have been reported previously in cooked dairy products, including acetic acid, 2,3 butanedione, 4-hydroxy-2,5-dimethyl-3-furanone (Bertrand et al., 2011, 2015). Furthermore, many of the products are short chain carbonyl compounds which take part in common thermally induced reactions such as aldol condensation and Strecker degradation.

2.4.2.2 Strecker Degradation

The reaction of amino acids with alpha dicarbonyl compounds to produce the corresponding aldehyde is known as Strecker degradation (Strecker, 1862).

Figure 2.10 Representation of Strecker degradation.



(Strecker, 1862)

A reaction scheme for the reaction of leucine to give 3-methylbutanal is shown in figure 7. The same mechanism transforms various other amino acids into their respective aldehydes, as shown in table 2.

Table 2.2 Strecker aldehydes and their precursor amino acids

Amino acid	Aldehyde	Aroma of aldehyde
Leucine	3-Methylbutanal	Malt, Chocolate
Isoleucine	2-Methylbutanal	Malt
Valine	2-Methylpropanal	Malt
Alanine	Acetaldehyde	Ethereal
Phenylalanine	Phenylacetaldehyde	Honey
Methionine	Methional	Potato

2.4.2.3 Further Maillard reactions

Products of the early stages of the Maillard reaction and Strecker degradation react further to form a range of different odorants, including pyrroles, pyridines, pyrazines, imidazoles, oxazoles and thiazoles (Nursten, 1981).

While the Maillard reaction is complex with a great variety of different potential products, previous work has identified many key odorants and helped elucidate their mechanisms of formation. Studies involving the isotopic labelling of precursor compounds have been used to confirm the origins of many compounds (Shiota et al., 2015).

2.4.3 Cooked cheese flavour

Despite the relevance of cooked cheese flavour for the dairy industry, relatively little has been reported in the literature. Johnson and Olson (1985) reported the

browning of cheese during cooking, which is related to the level of reducing sugars in the cheese and due to the Maillard reaction (Wilhelm Henneberg, 1860).

Dumont et al (1976) reported volatiles detected in grated and oven cooked Gruyère. Gruyère is a popular cooking cheese in French cuisine which is often used in fondue, but is less commonly consumed in the UK and rest of the world. Their findings included aldehydes, ketones, alcohols, esters and sulfur compounds. They determined that the Maillard reaction contributes to formation of many volatiles during cooking in Gruyère, but their findings were unclear on the importance of lipid sources for cooked cheese flavour. The authors didn't undertake GC-O studies to investigate the importance of these volatiles to the aroma of cooked cheese, so their findings are unlikely to fully characterise cooked Gruyère aroma. Furthermore, their study did not consider non-volatile changes during cooking.

Bertrand et al studied flavour formation in a processed cheese model system during heating (Bertrand et al., 2011, 2015). Processed cheese is a popular manufactured product in the United States of America, containing a portion of "real" cheese but with substantially higher moisture and lower protein content. For example, the processed cheese studied by Bertrand et al contained 60 % moisture and 12 % protein, compared to 30-40 % and 24%-43 % respectively in the mild Cheddars studied in this thesis. The work of Bertrand et al included GC-O and detected several odorants in cooked processed cheese, including 2,3 butanedione, furaneol, maltol, 2-acetyl-pyrazine and dimethyl trisulfide. However, the cooking conditions employed were much less harsh than those typical for pizza cooking or baking, reaching just 150 °C for a total cook of 5 minutes.

Henneberry et al (2015) studied the sensory characteristics and volatile profiles of unheated and heated mozzarella cheese, specifically the effect of reduced fat, salt and calcium levels in the cheese. The heating conditions were mild (10 minutes in a water bath at 95 °C), conditions likely to have induced melt rather than browning in the cheese. Ketones, alcohols, fatty acids and aldehydes were the majority of compounds detected. Their results focussed on the effect of reducing fat, salt and calcium on heated and unheated cheese respectively, rather than a direct comparison of the same cheese unheated and heated. It was shown that nonanal was significantly ($p < 0.05$) higher in the full-fat cheese than reduced fat when unheated, but not significantly different when heated. Phenylacetaldehyde was significantly ($p < 0.05$) higher in the reduced fat cheese than the full-fat cheese, both heated and unheated, and also correlated with reduced sensory liking scores and increased off-note scores.

Typical cooking conditions for cheese range from mild conditions (inducing melt rather than browning) to harsher conditions (e.g for traditional neopolitan pizza: 60-90 s at 485 °C (Ciarmiello & Morrone, 2016), for cheese-topped bakes: 20-45 min at 180-200 °C). Due to the mild cooking conditions used in previous studies, further studies are needed to confirm the aroma compounds responsible for more extensively cooked cheese flavour.

Bertrand et al also considered changes in glutamic acid during cooking, which was found to increase after heating at low temperatures, but decrease after heating at higher temperatures. (Bertrand et al., 2011, 2015). This thesis expands knowledge in this area to explore the concentration of a series of amino acids and γ-glutamyl peptides in a selection of cheeses, including low and medium-fat Cheddar, during cooking.

2.5 Focus for this thesis

From the knowledge outlined in section 2.4.3, the topic of cooked cheese flavour was still largely unexplored. To expand this topic, the scope of this work includes:

1. Characterisation of the volatiles in cooked cheese, including identification of odorants using GC-O.

2. Characterisation of selected non-volatiles in cooked cheese.

Selection of non-volatiles for study was based on either their role as tastants or precursors in uncooked cheese. Some cheese tastants, including salts and minerals, are unlikely to be affected by the temperatures reached during cooking. Other potential tastants, such as the various peptides found in cheese are still being characterised in uncooked cheese. Identification of all taste active peptides in cooked cheese is a large piece of work that is outside the scope of this project but would be an interesting topic for further exploration.

This work will be focussed on selected tastants that are both well characterised in uncooked cheese, and that we hypothesise are likely to be affected by lipid degradation or the Maillard reaction. These are amino acids, γ-glutamyl dipeptides, diketopiperazines, sugars and organic acids. Furthermore, each of these classes has the potential to act as precursors to volatile flavour development during the Maillard reaction.

3. Characterisation of the role of fat on both the volatile and non-volatile components of cooked cheese.

The concentration of fat in cheese may impact flavour formation during cooking. Lack of lipid precursors in low-fat cheeses may influence the formation of lipid

derived products during cooking, although literature data on fat concentration and cooked cheese flavour are scarce. Several compounds detected in heated dairy products are thought to be lipid derived, including lactones, fatty acids, ketones (e.g. 2-methylketones, 1-octen-3-one) and the alcohols formed by their reduction. This observation raises interesting possibilities about the formation of flavour during cooking of reduced and low-fat cheeses, which to the best of the author's knowledge has not been investigated previously.

In addition to the potential role of fat in cheese as a source of precursors for flavour development, the concentration of fat may affect cooked cheese flavour in other ways. The additional moisture, protein and carbohydrates in low-fat cheese may influence the formation of non-lipid derived compounds. Additionally, the presence of fat may play a role in cheese structure during cooking. If so, these structural changes may affect the generation of both odorants and tastants during the Maillard reaction, such as γ-glutamyl peptides, diketopiperazines and glutamate. The existence of fat taste, 'oleogustus' (the taste of fatty acids as a separate quality to their well-established aroma and mouthfeel properties), is still debated (Running et al., 2015) and therefore fatty acids are considered as contributors to aroma, but not to taste during this work.

2.6 Conclusion

In conclusion, cheese is a highly popular foodstuff in many countries globally and a key commodity for the EU and UK dairy industries. There is extensive prior research on uncooked cheese flavour, but the flavour of cooked cheese is yet to be characterised despite the prevalence of cheese as an ingredient in cooked dishes. By

combining existing knowledge on the flavour of uncooked cheese, lipid degradation and the Maillard reaction, the broad topic of cooked cheese flavour will be approached selectively by studying flavour compounds likely to be affected by cooking. Much of this work will focus on characterising the aroma of cooked cheese, but selected peptides, organic acids and sugars will also be compared in cooked and uncooked cheese. The role of fat in cooked cheese flavour is of specific relevance to the dairy industry and will also be a subject of study.

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Chapter 3 – Volatile characterisation of cooked cheese flavour.

Preface to chapter 3

This study explores the volatile compounds which contribute to cooked cheese flavour, including the odorants in cooked mature Cheddar using HS-SPME. This work relates to hypothesis 1.

Authors' contributions: As the main author on the study, I conducted the material preparation, the majority of the data collection and data analysis, performed GC-O and wrote the first draft of the manuscript. Fiyinfolu Makinwa assisted with data collection, analysis and the identification of odorants for GC-O, and produced part of this work in contribution to her Masters thesis. All authors contributed to the study conception and design and provided comments on the draft manuscript. Jane Parker was an additional GC-O panelist.

This chapter has been prepared for submission to journals, and will be submitted shortly.

Abstract

The aims of this work were to identify volatile compounds that contribute to the aroma of cooked cheese. Volatiles and odorants in cooked mature Cheddar were identified using a combination of SPME/GC-O and SPME/GC-MS. A selection of the odorants were quantitated in six cheeses, uncooked and cooked, (mature Cheddar, high-, medium- and low-fat mild Cheddar, traditional mozzarella and Parmesan). Many compounds showed significant differences between cooked and uncooked cheese; Strecker aldehydes, pyrazines and furanones were all significantly ($p < 0.05$) higher in cooked cheeses than in uncooked cheese, while ethyl esters (key odorants in uncooked cheese) were not detected in cooked Cheddar. Principal component analysis (PCA) demonstrated that fat concentration in mild Cheddar was positively correlated with formation of potential odorants (the Strecker aldehydes, methanethiol, 2-methylketones and fatty acids) upon cooking. Potential lipid precursors for these compounds are discussed.

3.1 Introduction

Cheese is an important commodity for the food industry. It is a key ingredient in a range of cooked dishes such as grilled cheese as toppings to bread, pasta and pizza dishes and melted, as in fondue. The aroma of uncooked cheese has been studied extensively and has been described as a balance between the concentrations of a wide variety of volatile compounds (Avsar et al, 2004 ; Carunchia Whetstine et al, 2006; Christensen and Reineccius, 1995; Frank et al, 2004; Drake et al, 2010; Suriyaphan et al, 2001; Zehentbauer and Reineccius, 2002; Wang et al, 2021). This ‘component balance theory’ (Kilcawley and O’Sullivan, 2007) states that the differences between cheese varieties can be attributed to the differences in the balance of the cheese odorants.

As a source of protein, sugars and fats, cheese has the potential to undergo heat-induced flavour and colour changes including the Maillard reaction, lipid oxidation and caramelisation. Despite the potential for flavour formation when cheese is cooked, the subject of cooked cheese aroma has received relatively little attention in the literature. Dumont et al. (1976) investigated the volatile compounds formed in gratinated Comté, and reported aldehydes, ketones and sulfur compounds. They reported that products of protein degradation had a clear role in the aroma of cooked cheese, while the contribution of fat and its breakdown products was less clear. Similarly Henneberry et al (2015) reported ketones, acids, aldehydes and alcohols in the volatile profile of heated mozzarella (95 °C).

Aside from cheese, aroma generation has been studied in other related cooked dairy products. Bertrand et al. (2011) identified 29 odour active volatiles in cooked processed cheese after heat treatment reaching a maximum temperature of 150°C for 7.5 min. However, the composition (especially the moisture content), structure and maturity of processed cheese differs substantially from the typical composition of cheese. Therefore, differences may be expected in the volatile compounds formed during the cooking of cheese and processed cheese.

This work expands on previous studies on cooked cheese flavour to focus on oven cooking. It is a comparison of the volatile compounds found in six cooked cheeses by headspace solid phase microextraction (HS-SPME), which has previously been used to investigate the profiles of uncooked cheese (Delgado et al., 2010; Frank, Owen & Patterson, 2004; Mondello et al., 2005; Lecanu et al., 2002; Henneberry et al, 2015). Three of the cheeses were commercially purchased (mature Cheddar, mozzarella and Parmesan) to represent a variety of different cheeses typically used in cooked dishes in the UK. Additionally, three mild Cheddars were produced with

varying fat content (~2-35%) from the same milk, to explore the role of fat content in flavour formation in cooked cheese. In part II of this two-part study (chapter 4), non-volatiles including sugars, amino acids and peptides were shown to decrease in cheese during cooking. It was hypothesized that these changes in non-volatile precursors would be accompanied with corresponding volatile changes. Furthermore, it was hypothesized that differences in the precursor pool between high and low-fat cheese would affect the formation of odorants during cooking.

3.2 Materials and methods

3.2.1 Materials

The following aroma standards were purchased: 2,3,5-trimethylpyrazine and cyclotene (IFF, Haverhill, UK); 3,5-dimethyl-2-ethylpyrazine, (E)-2-decenal (Oxford Organics, Hartlepool, UK); hexanal, 2,3-butanedione, 2-heptanone, 2-methoxyphenol, dimethyl disulfide, dimethyl trisulfide, 2-methylbutanal, 3-methylbutanal, phenylacetaldehyde, 4-hydroxy-2,5-dimethyl-3-furanone, 2/3-methylbutanoic acid, hexanoic acid, methanethiol, 2-methylpropanal, (Z)-4-heptenal, octanal, 1-octen-3-one, 2-methyl-3-furanthiol, nonanal, (furan-2-yl)methanethiol, 2-isobutyl-3-methoxypyrazine, (E)-2-nonenal, 3-(methylsulfanyl)propanal(methional), 2-ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone and (E,E)-2,4-nonadienal (Sigma, Poole, UK); 2-acetyl-1-pyrroline (Aroma Lab, Munich, Germany), All other chemicals used were obtained from Sigma Aldrich Ltd. (Gillingham, UK). The internal standard was 0.25 mg/L isopropylpyrazine in saturated sodium chloride water solution. Chemicals used for amino acid analysis were obtained from the EZ:FAAST kit from Phenomenex (Torrance, CA, USA).

3.2.2 Cheeses

Three cheeses were purchased from a supermarket: mature Cheddar (Ched), fresh mozzarella (Mozz) and Parmesan (Parm). Cheddar, mozzarella and Parmesan are all commonly used in the UK and vary considerably in terms of maturity. Typical aging periods for these cheeses are shown in table 3.1. Three mild Cheddar cheeses of differing fat content, low-fat (LF, 2 % fat), medium fat (MF, 22 % fat), high-fat (HF, 35 % fat), were made at the University of Reading's pilot plant facility (Reading, UK) as described in 3.2.3, and were included to determine the effect of fat content on formation of cooked cheese odorants. See Appendix 12 for full compositional comparison.

Table 3.1 Cheeses studied, their abbreviations and aging periods.

Cheese	Abbreviation	Aging period
mozzarella	mozz	<1 months (typical)
Parmesan	parm	> 22 months (manufacturers description on packaging)
mature Cheddar	ched	9 months (typical)
mild Cheddar	HF (high-fat), MF (medium fat), LF (low-fat)	3 months

(McSweeney, 2017)

3.2.3 Cheesemaking

Milk was obtained from the University of Reading dairy herd of Holstein Friesians (CEDAR, University of Reading, UK). Their diet (expressed per cow per day) included concentrate blend (9.5 kg), hay (1.0 kg), grass silage (19.0 kg), maize silage (24.0 kg), Trafford Gold (cow feed based on wheat byproducts) (4.0 kg), fat (0.1 kg),

salt (0.1 kg), limestone flour (0.1 kg), minerals (0.03 kg). The milk was pasteurised (73 °C for 15 s) using a continuous pasteuriser with plate heat exchangers operating at approximately 300 L/h. The fat was separated from the milk using a disk bowl separator and then recombined to produce standardised milk with fat contents of 0.10 %, 2.71 % and 3.9 % respectively in order to produce Cheddar cheese with low, medium and high-fat contents respectively.

Cheddar cheese making was carried out in 100 L jacketed cheese vats. Standardised milk (100 L) was heated to 30 °C in cheesemaking vats, when 0.1 g/L starter culture R604 (Chr. Hansen, Hungerford UK) was added. The initial pH of the milk was 6.75 ± 0.01. Once the pH decreased to 6.65 ± 0.02, Chymosin, (26 g ± 1.12, CHYMAX, Chr. Hansen, UK) was added to initiate coagulation. After 60 min (pH 6.60 ± 0.03) the coagulum was cut. Scalding was then initiated by increasing the temperature to 38 °C over 40 min and holding it at this temperature for 50 min.) Following the scalding, the whey was drained from the curd which was then Cheddared. Once the pH reached 5.30 ± 0.01 (approximately 60 minutes) the curd was milled and salted (2% w/w) and then pressed in moulds for 24 h. Compositional data were measured for the three cheeses, as described in appendix 12. They were then matured at 8 °C for 3 months (producing mild Cheddars) which were used for analysis. Additionally small portions of the cheeses were further aged to 6, 9, 12, 18 and 24 months for amino acid and γ-glutamyl peptide analysis. Other analyses were not performed on samples aged 6 months or longer due to the limited quantity of each sample. The cheese was cut into pieces (approximately 250 g), vacuum-packed and stored at -20 °C until use. The final weights of high, medium and low-fat Cheddar were 10.2, 8.8 and 6.2 kg respectively.

3.2.4 Cheese Sample Preparation

Grated cheese (50 g) was spread evenly on a glass petri dish (90 mm diameter x 10 mm depth) and baked in a GC Oven (Hewlett Packard 5890 Series II) at 180 °C for 20 min. It was then cooled to room temperature, immersed in liquid nitrogen, and ground in a coffee grinder (Quest, Liverpool UK) to obtain a fine powder. The cooked cheese was extracted as soon as it reached room temperature to minimise losses of volatile compounds, any volatile losses during cooling were assumed to be negligible compared to the losses occurring during oven cooking.

3.2.5 GC-MS Analysis

In triplicate, powdered cheese (all six cheeses, uncooked or cooked) (1.5 g ± 0.1 g) was transferred into a 20 mL headspace vial (Supelco, Munich, Germany) sealed with a screw top lid. Internal standard solution isopropylpyrazine (0.25 mg/L, in saturated aqueous sodium chloride solution, 1.5 mL) was added and the vial was vortexed for 30 s. The samples were incubated (50 °C, 10 min) using a CTC 120 autosampler (Agilent, Santa Clara, USA). The volatiles in the headspace were extracted (50 °C, 20 min) using a Carboxen/DVB/PDMS SPME fibre (Supelco, Munich, Germany). The temperature of SPME incubation and extraction and the fiber phase are important considerations in method choice. In this case they was selected to align with methodology described in the literature (Delgado et al, 2010) to allow for effective capture of volatiles, while minimising artefact formation. The fibre was desorbed at 250 °C for 20 min in the injection port of a 7890A GC coupled to a 5975C Inert MS detector (both Agilent, Santa Clara, USA) fitted with a ZB-5MSi column (30m, 0.25mm, 1μm) (Phenomenex, Torrance, USA). The oven temperature started at 50 °C which was held for 2 min, followed by 6 °C/min ramp to a maximum temperature

of 300 °C which was held for 15 min. The carrier gas was helium, at a constant flow rate of 0.9 mL/min. Mass spectra were recorded in the electron impact mode at an ionisation voltage of 70 eV and source temperature of 250 °C.

An alkane standard C5-C25, 10 mg/L in diethyl ether was used as a reference for calculation of the LRIs. Compounds were identified using the MS mass spectra from NIST 11 library and confirmed by comparison to LRIs of authentic compounds or using LRIs from the NIST Chemistry WebBook.

3.2.6 GC-O Analysis

Powdered cheese was prepared as described in section 3.2.5, but without addition of the internal standard solution. SPME extraction was performed manually using a waterbath to incubate the samples (50 °C, 10 min). The volatiles in the headspace were extracted (50 °C, 20 min) using a manual Carboxen/DVB/PDMS SPME fibre (Supelco, Munich, Germany). Analysis was conducted on a 7890B GC system (Agilent, Santa Clara, USA) fitted with an ODO II olfactory detector from SGE Analytical (Ringwood, Victoria, Australia). A HP-5MS Ui column (30 m x 0.25 mm x 0.25 μm, Agilent Technologies) was fitted. The carrier gas was helium at 2 mL/min. The injection port was held at 250 °C, after injection of the manual fibre, the oven was held at 40 °C for 2 min followed by an initial ramp of 4 °C/min until 200 °C was reached then a ramp of 8 °C/min until 300 °C was reached. The final temp was then held for 8 min. The flow from the column was split between an FID detector and a sniffing port 1:1, followed by two untreated silica-fused capillaries of the same dimensions (1 m, 0.32 mm i.d.). The flow to the sniffing port was diluted with a moist make up gas. The FID detector was kept at 250 °C with flowrates of 40 mL/min hydrogen, 400 mL/min air, and 9 mL/min nitrogen. GC-O analysis was conducted in

duplicate by two expert sniffers describing the odors in their own words and recording the retention time and the intensity of each odour on a scale of 1-10 (very weak to very strong). An alkane standard C5-C25 (1 mL), 10 mg/L in diethyl ether was used as a reference for calculation of the LRIs. Compounds were identified on the basis of odour (The Good Scents Company Website (TGSC, 2018) and verified by comparison to LRIs of authentic compounds and LRIs from the NIST Chemistry WebBook library, and MS in the GC-MS chromatogram. GC-O was repeated by one sniffer on a different column phase (DB-FFAP polar column (30 m 0.25 mm I.D., 0.25 µm film thickness), Phenomenex, Macclesfield, UK) otherwise using the same extraction and chromatographic conditions.

3.2.7 Amino Acid Analysis

The method described by Toelstede *et al.*, (2009) was used to prepare a water-soluble extract of the six uncooked cheeses. The extracts were freeze-dried and ground into a powder, and prepared for amino acid analysis using the EZ:FAAST system. Analysis was conducted on an Agilent Technologies 6890N GC system coupled to an Agilent 5975 inert XL Mass Selective detector. The oven was fitted with a ZBAAA GC column. For full details and discussion on amino acid analysis, see chapter 4.

3.2.8 Semi-quantitation

The volatiles were semi-quantitated according to the equation below:

Conc. (A) = (single ion peak area (A)* factor (A)) / (single ion peak area (IS) * factor (IS)) * conc. (IS).

where A represents each analyte. Semi quantitation was performed using the peak areas of a single selected ion per analyte from the GC-MS chromatogram, relative to that of the internal standard. The ‘factor’ was used to correct the peak area of the

single ion to the peak area of the full scan chromatogram, and was calculated from a clean spectrum for each analyte using the following equation:

Factor (A) = peak area (A) / single ion peak area (A).

3.2.9 Statistical interpretation

The determination of statistical significance was performed using SPSS (IBM, version 25). The significance of differences between the data were determined using a multivariate linear model and a Post-Hoc test of multiple comparisons for observed means using a Tukey HSD with an alpha of 0.05. The principal component analysis (PCA) was performed on the data in table 3.2 using XLSTAT (version 2019.4.2.63912). The statistical interpretation for the amino acid quantitation was performed using XLSTAT ANOVA followed by a Tukey HSD with an alpha of 0.05.

3.3 Results and Discussion

3.3.1 Identification of odorants in cooked Cheddar

SPME-GC-O was used to identify odorants in cooked mature Cheddar. This sample was chosen as a typical cheese used in cooked applications in the UK. Odorants detected in cooked Cheddar are shown in table 3.2. Semi-quantitative results for cooked Cheddar odorants in all six cooked cheeses are shown in table 3.3, while semiquantitative results for a broader selection of compounds in all cheeses (both cooked and uncooked) can be found in appendices 1 and 3.

Of the 36 odorants detected, 8 have not been reported previously in GC-O data from uncooked Cheddar (3-methyl-2-butene-1-thiol, 2-heptanone, (furan-2-yl)methanethiol, 3-methyl-1,2-cyclopentanedione (cyclotene), 3-ethyl-2,5-dimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, 2-methyl-3-methyldithiofuran and (E)-2-decenal). These compounds are likely to contribute to differentiation of cooked

Cheddar from uncooked Cheddar aroma. The most intense odorants were 3-methylbutanal, 2-methylpropanal, 2-methylbutanal, (Z)-4-heptenal, methional, (furan-2-yl)methanethiol, 2-methyl-3-furanthiol, methanethiol, 2-methyl-3-methyldithiofuran and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone by assessor intensity score. The Strecker aldehydes, 3-ethyl-2,5-dimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, cyclotene and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone are known products of the Maillard reaction which have been reported in other cooked foods, but have not previously been related to aroma in cooked cheese.

The odorants detected in cooked Cheddar were semi-quantitated using GC-MS data in all six cheeses (see table 3.3 for cooked cheese data, and appendix 3 for uncooked data). In general, trends were observed in the behaviour of compounds according to their formation pathway. The graphs in figure 3.1 outline semi-quantitative data for one compound from each formation pathway in the cooked and uncooked cheeses respectively, as an example of the broader trends.

Three pyrazines with roasted aromas, (trimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine) were detected as odorants in cooked Cheddar, alongside a further six pyrazines which were detected by SPME-GC-MS. Each pyrazine was significantly ($p < 0.05$) higher in several cooked cheeses than in their uncooked counterparts, an example of the pyrazine data in cooked cheese is shown for 2-ethyl-3,5-dimethylpyrazine in figure 3.1. These pyrazines are likely to form during the Maillard reaction via α-amino carbonyl compounds generated during Strecker degradation (Weenan et al., 1994).

Dumont et al. (1976) reported the presence of a number of pyrazines (methylpyrazine, 2,3-dimethylpyrazine, 2,5 dimethylpyrazine, trimethylpyrazine, 3-

ethyl-2,5-dimethylpyrazine, tetramethylpyrazine, 2-ethyl-3,5,6-trimethylpyrazine, triethylpyrazine) in cooked Gruyère. Given these findings, pyrazines are likely to be important compounds in cooked cheese aroma.

2-Isobutyl-3-methoxypyrazine was also found to be an odorant in cooked Cheddar, however, it is not formed through the same mechanistic pathway as the other pyrazines reported. 2-Isobutyl-3-methoxypyrazine was not included in the semi-quantitative results as it could not be detected by SPME-GC-MS. This compound has been reported to contribute to earthy flavour in uncooked Cheddar by Suriyaphan et al. (2001). They reported higher concentrations of 2-isobutyl-3-methoxypyrazine near the rind and hypothesised that it may form by the action of cheese molds.

Three odorants (cyclotene, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone and 2-ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone) detected in cooked Cheddar are known to form from the sugar moiety during the Maillard reaction. Cyclotene and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone were present in high enough concentration to be semi-quantitated from SPME-GC-MS data in each of the cooked cheeses.

Neither were detected in any of the uncooked cheeses, although 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone and 2-ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone have previously been reported as odorants in uncooked Cheddar (see table 3.2 for references). Given their previously reported importance in uncooked Cheddars, and higher concentration in cooked cheeses, all three compounds are likely to be important to the aroma of cooked cheese.

Five Strecker aldehydes (2-methylpropanal, 3-methylbutanal, 2-methylbutanal, methional and phenylacetaldehyde) were all reported as odorants in cooked Cheddar, and were shown to be higher in cooked cheeses than in uncooked by SPME-GC-MS.

1802 Table 3.2 Odorants detected in cooked mature Cheddar by SPME-GC-O

No.	Compound	Odour	Intensity	LRI (GC-O) DB-5	LRI (GC-O) FFAP	Identity based on [a]	References [b]
1	methanethiol	rotting	5	< 600		Odour, lri, MS	E, H
2	2-methylpropanal	chocolate	8	< 600	< 800	Odour, LRI, MS	A
3	2,3-butanedione	butter	6	605	985	Odour, LRI, MS	A, B, D, E, F, G
4	3-methylbutanal	chocolate	10	653	911	Odour, LRI, MS	A, D, E, G
5	2-methylbutanal	chocolate	8	664		Odour, LRI, MS	D
6	dimethyl disulfide	savoury vegetal	5	718	1047	Odour, LRI, MS	H
7	butanoic acid	sweaty	7	777	1624	Odour, LRI, MS	A, C, D, E, F, G
8	hexanal	green	3	804	1077	Odour, LRI	A, B, F, G
9	3-methyl-2-butene-1-thiol	cannabis	6	823		Odour, lri	
10	3-methylbutanoic acid	sweaty	5	844		Odour, LRI, MS	C, E
11	2-methyl-3-furanthiol	meaty	5	867		Odour, lri	B, F
12	2-heptanone	fruity, blue cheese	5	898	1178	Odour, LRI, MS	
13	(Z)-4-heptenal	lamb fat	8	904		Odour, LRI	B, F, G
14	methional	potato	8	909	1450	Odour, LRI, MS	A, B, C, D,E, F, G, H
15	(Furan-2-yl)methanethiol	coffee	7	913		Odour. lri	
16	2-acetyl-1-pyrroline	basmati rice	5	926	1333	Odour, LRI	B, E, G
17	hexanoic acid	sweaty	3	970	1838	Odour, LRI, MS	D, E, F
18	dimethyl trisulfide	sulfurous, pungent	6	974	1373	Odour, LRI, MS	A, B, C, E, F, G, H
19	1-octen-3-one	mushroom	4	962	1306	Odour, LRI	A, B, C, D, E, F, G
21	trimethylpyrazine	pyrazine-like	5	1005		Odour, LRI, MS	H

1803

22	cyclotene	biscuit	4	1029	1822	Odour, lri, MS	
23	phenylacetaldehyde	floral, honey	6	1047	1639	Odour, LRI, MS	A, B, C, F
24	4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (furaneol)	candy floss	7	1084	1996	Odour, lri, MS	A, C, E, F, G
25	4-methylphenol (p-cresol)	faecal	5	1081		Odour, lri, MS	B, C
26	3-ethyl-2,5-dimethylpyrazine	pyrazine-like	3	1083	1443	Odour, LRI, MS	
27	2-ethyl-3,5-dimethylpyrazine	pyrazine-like	5	1088		Odour, lri	
28	2-methoxyphenol (guaiacol)	smoky, fire	4	1101		Odour, LRI	C
29	nonanal	fruity, fatty	5	1110		Odour, LRI, MS	A, F, G
30	2-Ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone (ethyl furaneol)	sweet, maltol-like	4	1142		Odour, LRI	E, G
31	(*E*,*E*)-2,6 nonadienal	fatty, aldehyde	6	1153		Odour, LRI	B, F, G
32	(*E*)-nonenal	fatty sheets	4	1165	1527	Odour, LRI	B, F, G
33	2-methyl-3-methyl dithiofuran	meaty	4	1178		Odour, lri	
34	2-isobutyl-3-methoxy pyrazine	pepper	3	1217		Odour. LRI	B, C, G
35	(*E*,*E*)-2,4-nonadienal	fatty	4	1221		Odour, lri	G
36	(*E*)-2-decenal	fatty sheets	4	1255		Odour, LRI	

[a]Compounds were identified by verifying odour descriptors with The Good Scents Company Website (TGSC, 2018) (Odour), comparison of mass spectra with NIST 11 library (MS) and comparison of LRIs with authentic standards on a DB-5 column (LRI) or the NIST Chemistry WebBook (lri). [b]previously reported in cooked processed cheese: A - Bertrand et al. (2011), uncooked cheese B – Avsar et al. (2004), C– Suriyaphan et al. (2001), D- Christensen & Reineccius (1995), E– Drake, Miracle & McMahon (2010) , F– Carunchia Whetstine et al. (2006), G – Zehentbauer & Reineccius (2002), H– Frank, Owen & Patterson (2004).

1809 Table 3.3: A selection of odorants from cooked mature Cheddar, quantitated across all six cooked cheeses.

	Cooked Cheddar	Cooked HF	Cooked MF	Cooked LF	Cooked Mozzarella	Cooked Parmesan
Pyrazines						
trimethylpyrazine	1.2 ^{d}	0.34 ^{c}	0.3 ^{c}	0.21 $^{a\,b\,c}$	0.26 $^{b\,c}$	0.16 $^{a\,b\,c}$
3-ethyl-2,5-dimethyl-pyrazine	0.58 ^{c}	0.14 ^{b}	0.13 ^{b}	0.16 ^{b}	0.088 $^{a\,b}$	0.17 ^{b}
Sulfur compounds						
methanethiol	0.58 ^{d}	0.32 $^{b\,c\,d}$	0.21 $^{a\,b\,c}$	0.08 $^{a\,b}$	0.01 ^{a}	0.39 $^{c\,d}$
dimethyl disulfide	0.4 $^{a\,b}$	0.65 $^{a\,b\,c}$	0.31 $^{a\,b}$	0.86 $^{b\,c}$	0.24 ^{a}	1.2 ^{c}
dimethyl trisulfide	0.3 $^{a\,b}$	0.23 ^{a}	0.14 ^{a}	0.73 $^{b\,c}$	0.09 ^{a}	0.89 $^{b\,c}$
Strecker aldehydes						
2-methylpropanal	5.9 ^{d}	3.2 ^{c}	2.3 $^{b\,c}$	1.5 $^{a\,b}$	0.15 ^{a}	5.9 ^{d}
3-methylbutanal	33 $^{b\,c}$	45 ^{c}	31 $^{b\,c}$	16 $^{a\,b}$	0.87 ^{a}	13 ^{a}
2-methylbutanal	16 ^{b}	3.4 ^{a}	2 ^{a}	3.1 ^{a}	0.5 ^{a}	22 ^{c}
methional	0.79 ^{d}	0.55 ^{c}	0.37 ^{b}	0.11 ^{a}	0.01 ^{a}	0.42 ^{b}
phenylacetaldehyde	3.9 $^{c\,d}$	4.1 ^{d}	4.6 ^{d}	2.6 $^{b\,c}$	0.08 ^{a}	2.39 ^{b}

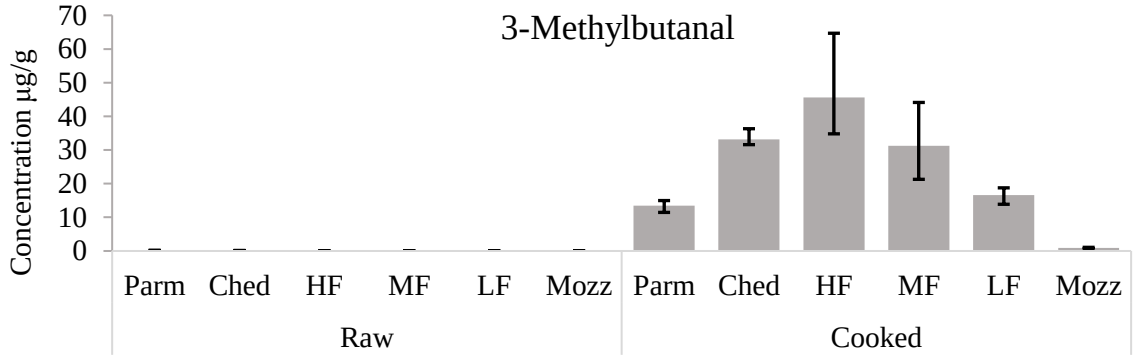
	Cooked Cheddar	Cooked HF	Cooked MF	Cooked LF	Cooked Mozzarella	Cooked Parmesan
Acids						
butanoic acid	4 $^{c d}$	2.5 $^{a b c}$	3 $^{a b c}$	0.4 ^{a}	0.11 ^{a}	18.5 ^{e}
3-methylbutanoic acid	0.11 $^{d e}$	0.05 $^{b c}$	0.05 $^{b c}$	0.01 $^{a b}$	0.01 ^{a}	0.14 ^{e}
hexanoic acid	1.5 $^{a b c d}$	0.86 $^{a b c}$	1.5 $^{a b c d}$	0.58 $^{a b}$	0.09 ^{a}	12 ^{e}
Other compounds						
2-heptanone	10 ^{c}	12 ^{c}	13 ^{c}	1.3 ^{a}	5.9 ^{b}	5.7 ^{b}
2,3-butanedione	1.4 ^{a}	1.7 ^{a}	1.6 ^{a}	1.3 ^{a}	2.5 ^{b}	1.2 ^{a}
3-methyl-1,2-cyclopentanedione	0.21 ^{c}	0.09 ^{b}	0.06 ^{b}	0.01 ^{a}	0.01 ^{a}	ND ^{a}
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	0.09 ^{d}	0.05 ^{c}	0.04 $^{b c}$	0.01 ^{a}	0.01 $^{a b}$	0.02 $^{a b}$
4-methylphenol	0.01 ^{e}	0 ^{c}	0 $^{a b c}$	0 $^{b c}$	0 $^{a b}$	0.01 ^{d}

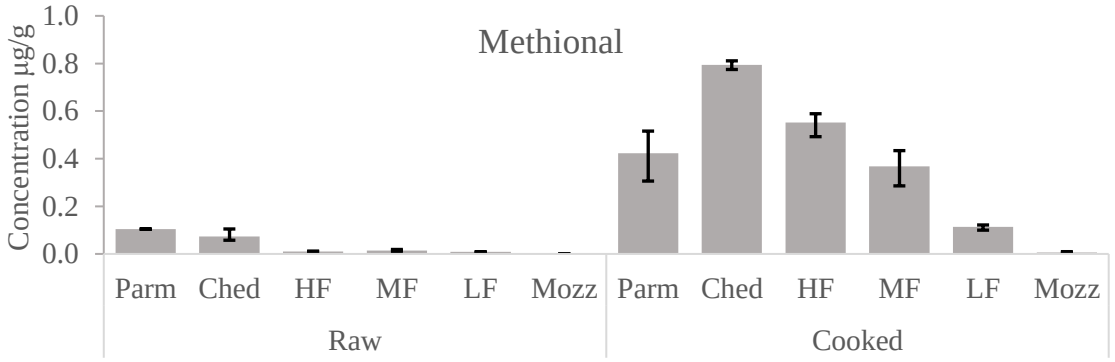
1810

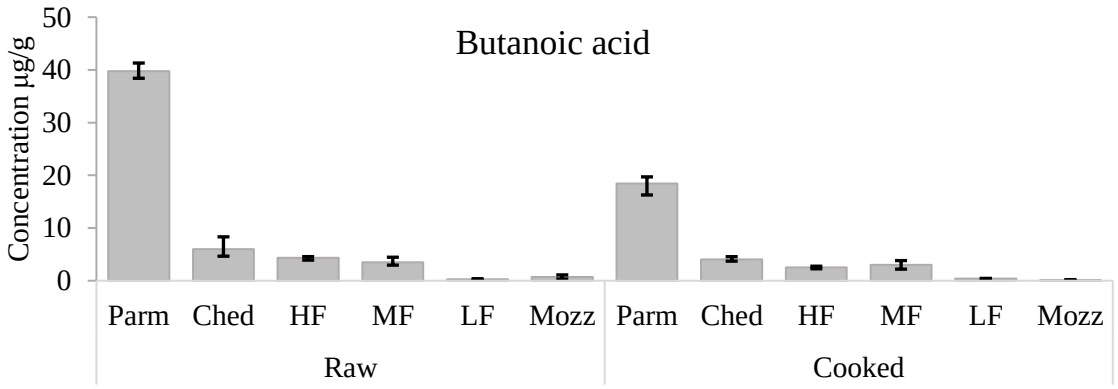
1811 Quantitation of selected odorants (μg/g) from cooked Cheddar cheese in a range of cooked cheeses. ND indicates compounds which

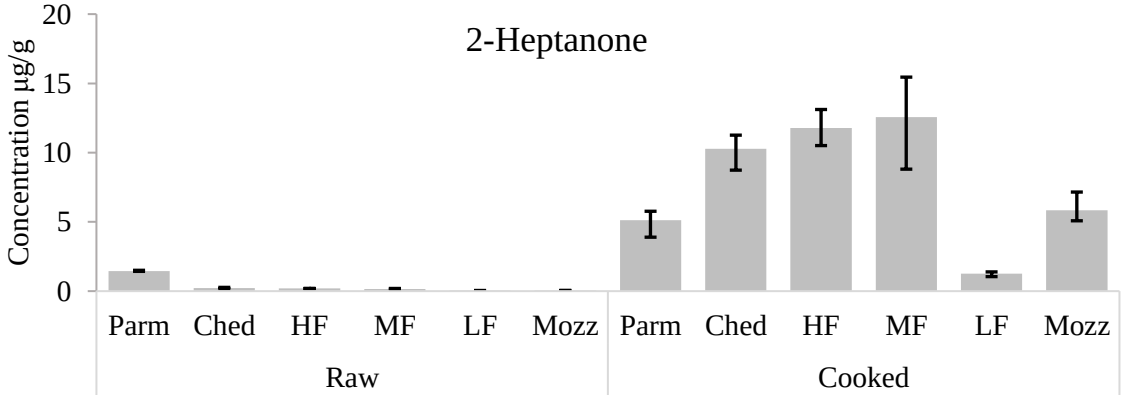
1812 were not detected.

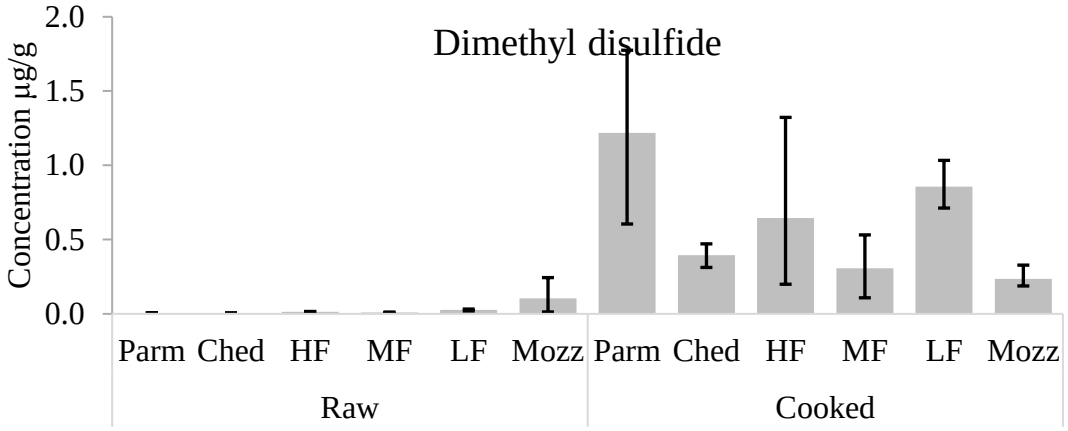
Figure 3.1 Bar graphs of semi-quantitative results from a range of compounds derived from different formation pathways in six uncooked and cooked cheeses.











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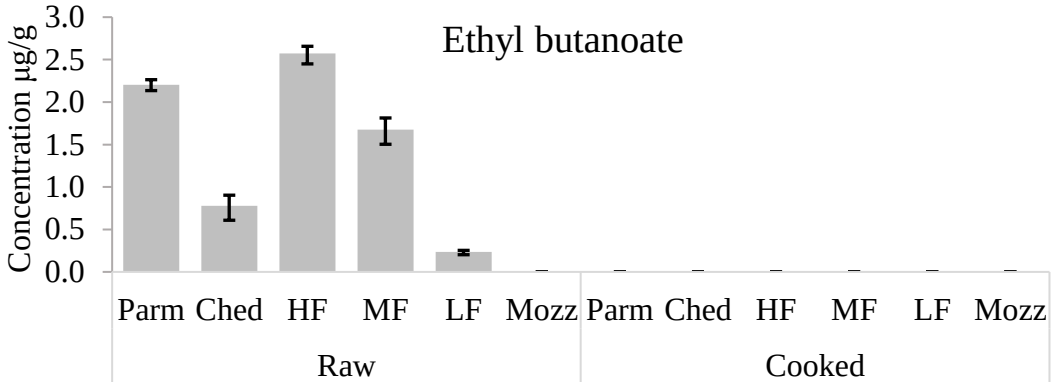

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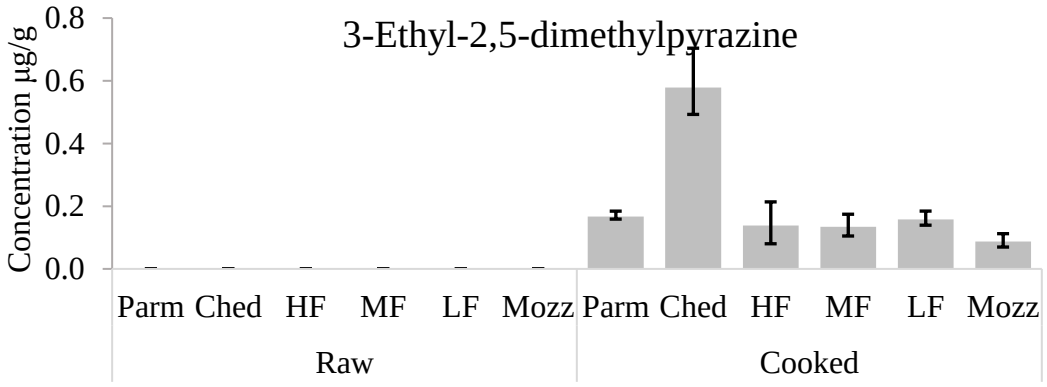

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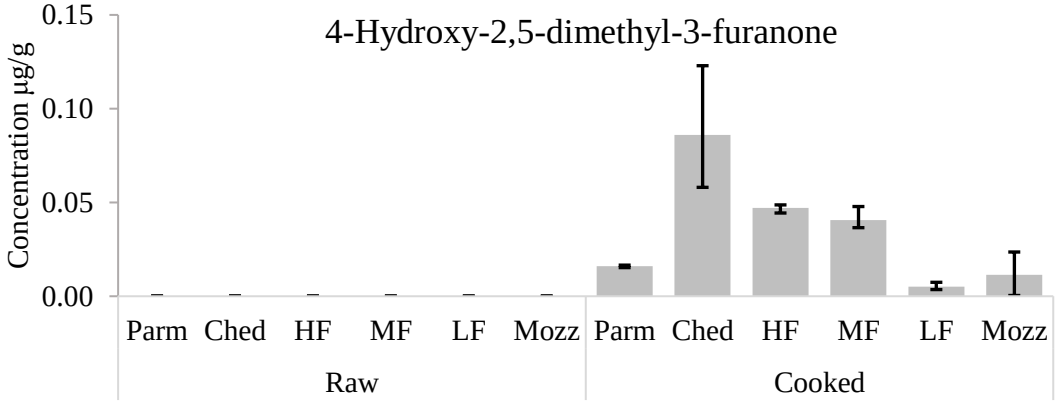

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1823 Error bars represent the range of the replicates. Abbreviations are shown in table 3.1.

Figure 3.1 shows the relative concentration of both 3-methylbutanal and methional in uncooked and cooked cheeses, representing the trend for formation of Strecker aldehydes during cooking. Formation of Strecker aldehydes is a pathway of the Maillard reaction, in which amino acids are converted into their corresponding aldehydes (2-methylpropanal, 3-methylbutanal, 2-methylbutanal, methional and phenylacetaldehyde respectively). The corresponding amino acids for each of these Strecker aldehydes were quantitated in the uncooked cheeses (data shown in appendix 2). Their concentration increased with the typical length of aging of the cheeses (mozzarella < mild Cheddars < mature Cheddar < Parmesan) (see table 3.1). The formation of Strecker aldehydes has been reported in cooked Gruyère (Dumont et al. , 1976) and cooked processed cheese (Bertrand et al. , 2011). Although Strecker aldehydes are found in uncooked cheese, their significantly ($p < 0.05$) higher concentration in cooked cheeses suggests they are important to flavour development during cooking of cheese.

Of the eight sulfur compounds identified as odorants during GC-O, only methanethiol, methional, dimethyl disulfide and dimethyl trisulfide were present in high enough concentration to be semi-quantitated by SPME-GC-MS. Each was significantly ($p < 0.05$) higher in cooked cheeses than in their uncooked counterparts, as shown for methional and dimethyl disulfide in figure 3.1. Of those odorants too low to quantitate by SPME-GC-MS, 2-methyl-3-furanthiol, (furan-2-yl)methanethiol and 2-methyl-3-methyl dithiofuran have all been reported previously in cooked meat (Mottram, 1998) and are thermally derived from cysteine or thiamine breakdown.

Methanethiol, methional and dimethyl trisulfide have long been considered key odorants in uncooked Cheddar. Methional and dimethyl trisulfide were also reported by Bertrand et al. (2011) as odorants in cooked processed cheese. Methanethiol forms

from breakdown of methional during the Maillard reaction (Belitz, Grosch and Schieberle 2009), and there was a correlation between the level of methanethiol and methional in the cooked cheeses. Dimethyl disulfide and dimethyl trisulfide are formed from the oxidation of methanethiol, although there was no correlation between the level of these sulfides and methanethiol detected in the cooked cheese.

2-Heptanone was the only 2-methylketone found to be odour active in cooked Cheddar, however, five other 2-methylketones were also detected by SPME-GC-MS. The 2-methylketones were all found at significantly ($p < 0.05$) higher concentrations in the cooked cheeses than their uncooked counterparts, except for low-fat mild Cheddar. 2-Methylketones have been shown to form upon heating of milk fat from esterified β-ketoalkanoic acids in glycerides via an hydrolysis and decarboxylation reaction (Calvo and de la Hoz, 1992).

Butanoic acid and hexanoic acid were all detected in cooked cheese by GC-O, and were quantitated along with several other fatty acids using SPME-GC-MS. They were generally lower, in some cases significantly ($p < 0.05$) so, in the cooked cheeses compared to their uncooked counterparts. Figure 3.1 shows an example of these data for butanoic acid. This suggests that degradation or volatile loss of short chain saturated fatty acids occurs when cheese is cooked.

Fatty acids, especially butanoic and hexanoic acid have been shown to be key odorants and some of the most abundant volatile compounds in uncooked cheese (Christensen and Reineccius (1995), Drake, Miracle and McMahon (2010) , Carunchia Whetstine et al. (2006)). Bertrand et al. (2011) also reported butanoic acid to be an odorant in cooked processed cheese. Our findings suggest that fatty acids play a role in cooked cheese aroma, but they were among the few odorants to decrease

in concentration during cooking. This suggests they may play a lesser role in cooked cheese aroma than other volatiles which increased significantly ($p < 0.05$) in concentration during cooking .

No esters were detected by GC-O or GC-MS in cooked Cheddar. This is in contrast to previous studies on the aroma of uncooked Cheddar, in which esters such as ethyl butanoate, ethyl hexanoate and ethyl acetate have been reported as fruity odorants (Suriyaphan et al., 2010; Avsar et al., 2004, Christensen and Reineccius, 1995). Esters were only detected in the uncooked cheeses, as shown for ethyl butanoate in figure 3.1. Esters are known to undergo hydrolysis at higher temperatures, furthermore, esters are volatile and have low boiling points. Both hydrolysis and volatile loss are likely to contribute to loss of esters during cooking. Unlike other highly volatile compounds in cheese (e.g 3-methylbutanal, methanethiol), esters are also unlikely to be replaced by generation in thermally induced reactions when lost.

In conclusion, we report a number of differences in the presence of low odour threshold odorants in cooked Cheddar compared to those previously reported in uncooked Cheddar. Most notably these include the presence of 3-ethyl-2,5-dimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine and cyclotene and the lack of ethyl esters). Additionally, while the majority of the odorants detected in cooked Cheddar have been previously reported in uncooked Cheddar (see table 3.2), the semi-quantitative results demonstrate large differences in concentration between the uncooked and cooked cheeses. Strecker aldehydes, sugar derived compounds such as cyclotene and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, sulfur compounds and 2-methylketones were all substantially higher in cooked cheeses than their uncooked counterparts, suggesting that they contribute to differentiating cooked cheese from uncooked cheese aroma.

Component balance theory is often used to describe the variance in flavor of different cheese varieties by their relative balance of a range of volatiles (Kilcawley and O'Sullivan, 2007). Our findings suggest that component balance may also apply for cooked cheeses, as cooking cheese altered the balance of aroma compounds, as well as leading to some formation and loss of odorants.

3.3.2 Differences in volatile composition of cooked cheese by cheese type

Principal component analysis (PCA) was performed to generate a representation of the data as a smaller set of variables (principal components). This allowed further exploration of the balance of volatiles in different varieties of cooked cheese, the clusters within the data and the relationships between the variables.

PCA was performed on the compounds quantified in table 3.3 across all six cooked cheeses. The first two principal components accounted for 48.2% and 27.6% of the variance between the samples respectively, such that the total variance accounted for was 75.8%. From examination of the rotated component matrix scores, the variables which influenced separation in each of the principal components (PCs) were determined. Component 1 (PC1) related to a number of Maillard reaction products (pyrazines, 3-methyl-1,2-cyclopentanedione, 4-methylphenol, 4-hydroxy-2,5-dimethyl-3-furanone, methanethiol and the Strecker aldehydes). Component 2 (PC2) was related to sulfur compounds (dimethyl disulfide and dimethyl trisulfide), 2-heptanone and 2,3-butanedione.

Figure 3.2 shows the PCA plot for the cooked cheeses, along with a plot of the variables. The cooked Parmesan samples were clustered in the top right of the PCA plot, separated from the other samples by PC-2. Comparison with the variable plot indicates that the separation is driven by higher levels of acids (hexanoic and

butanoic acid) and sulfur compounds (dimethyl trisulfide and dimethyl disulfide) in the Parmesan cheese than the other samples. Both of these differences may be related to the long aging process (typically 2 + years) used in Parmesan production. A high level of short chain fatty acids is typical in uncooked Parmesan, in which fatty acids are formed from triglycerides during aging in the process of lipolysis (Fox & McSweeney, 1996). Both dimethyl disulfide and dimethyl trisulfide are formed in cheese from the amino acid methionine (Belitz, Grosch & Schieberle, 2009). Free amino acids are also produced during the aging process via proteolysis, followed by further metabolism by starter and non-starter bacteria (Fox & McSweeney, 1996). Quantitation of a selection of amino acids in the uncooked cheeses (shown in appendix 7) confirms that the methionine concentration was significantly ($p < 0.05$) higher in Parmesan than the other cheeses.

Cooked mozzarella is clustered in the bottom left of the PCA, separated from the Cheddar datapoints by PC-1. This separation is related to the low concentration in mozzarella of many of the cooked cheese odorants including the Strecker aldehydes, short chain fatty acids, and the high concentration of 2,3-butanedione compared to the other cooked cheeses. As a fresh cheese, the progress of lipolysis and proteolysis are limited, generating fewer short chain fatty acids and amino acids, explaining the low concentration of fatty acids and Strecker aldehydes when mozzarella is cooked. 2,3-Butanedione (diacetyl) is a dicarbonyl compound which is formed in uncooked cheese via glycolysis. It can also form in the early stages of the Maillard reaction from the breakdown of reducing sugars. As a fresh cheese, uncooked mozzarella contained more milk sugars (e.g lactose, galactose) than aged cheeses (see chapter 4 and appendix 6), which may account for the higher concentration of diacetyl in cooked mozzarella.

The Cheddar cheeses form three clusters on the PCA, separated mostly by PC-1. The mature Cheddar is clustered on the bottom right of the plot. This is related to higher concentrations of Strecker aldehydes, fatty acids and pyrazines than the other Cheddar samples. As with the Parmesan cheese, the higher Strecker aldehyde and fatty acid concentrations in mature Cheddar are related to the processes of proteolysis and lipolysis which occur during aging.

However, the pyrazine concentrations were higher in the cooked mature Cheddar than in the cooked Parmesan. Alongside amino acids, the other precursors to pyrazine formation are α-amino carbonyl compounds generated during Strecker degradation (Weenan et al., 1994; Divine et al., 2012). A possible theory to explain the low formation of pyrazines in Parmesan compared to Cheddar may be that, due to the extensive aging of Parmesan, reducing sugars and sources of dicarbonyl compounds formed from their breakdown are present at a lower concentration than other low and moderately aged cheeses. As both sugars/dicarbonyls and amino acids are required for formation of pyrazines, both long and short aging periods may be associated with low levels of pyrazine precursors. Mature Cheddar has a moderate aging period, typically close to 9 months, which may enable high pyrazine formation upon cooking due to the presence of both sugars/dicarbonyls and amino acids. The theory of low sugar-derived carbonyls in aged cheeses would still be consistent with the high formation of Strecker aldehydes by the Maillard reaction in cooked Parmesan, as lipid precursors may be contributing to their formation. It has been shown that lipid degradation can produce carbonyl precursors to Strecker aldehydes (Hildago & Zamora, 2016; Hildago & Zamora, 2019).

Comparison of mozzarella, Parmesan and Cheddar suggest that the age of a cheese may affect its aroma when cooked, both directly (due to differences in the aroma of

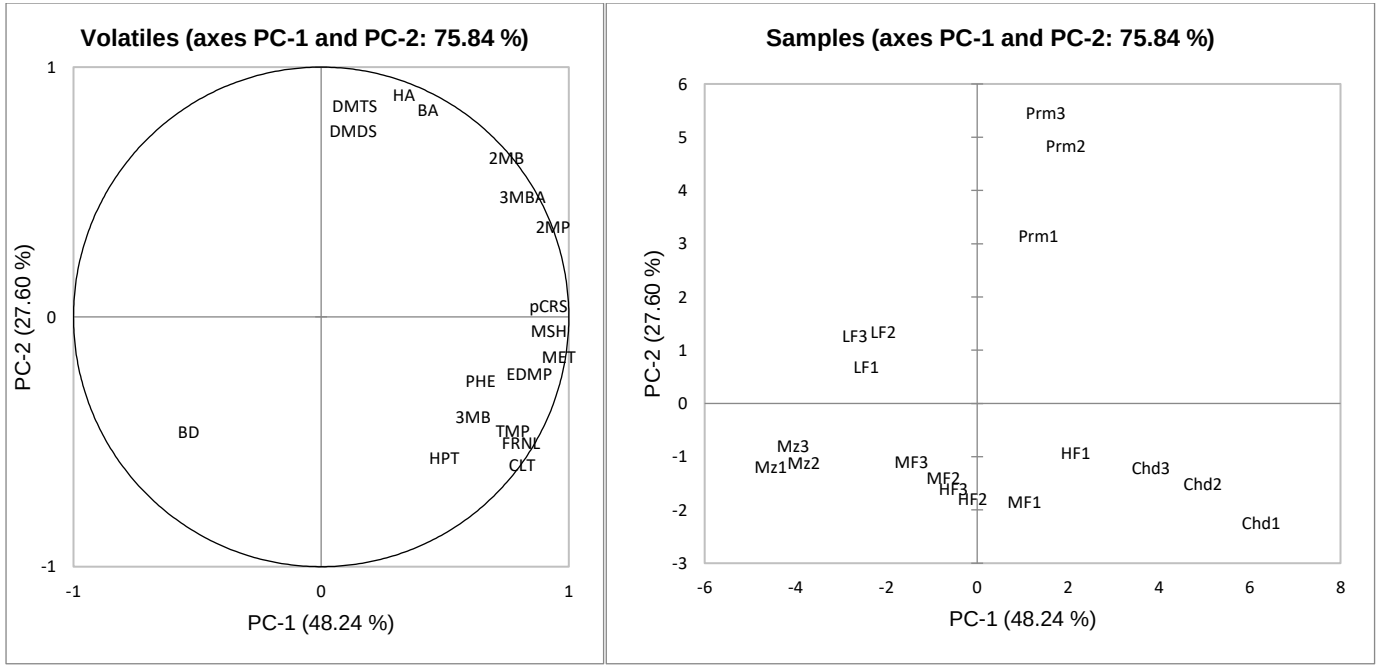
uncooked cheese which are maintained during cooking) and indirectly (by the formation or loss of precursors to aroma compounds during aging, which affects their conversion to aroma active compounds when the cheese is cooked).

3.3.3 The effect of fat content on cooked mild Cheddar flavour

Comparison of the three cooked mild Cheddars gives an indication of the role of fat in the development of flavour during cooking. In figure 3.2, the mild Cheddars were clustered into two groups distinct from the rest of the cooked cheese data. The low-fat mild Cheddar differed from the other mild Cheddars, while the medium and high-fat Cheddars were broadly similar and clustered together. In this study moderate reductions in fat concentration in Cheddars did not substantially affect volatile formation during cooking, while larger reductions had a much greater effect.

When comparing matrices of differing composition using headspace extraction techniques, it is important to consider how flavour release from the differing matrices may affect the results (Rincón et al., 2014). The pH and salt content of the matrix and the hydrophobicity of analytes influences how their release may be affected by differing fat content of the matrix (de Grazia et al, 2017). Highly hydrophobic compounds are likely to be less well released from high-fat matrices than from matrices of lower fat content, while anopposite trend would be expected from highly hydrophilic compounds. In this study an internal standard was used to account for some of the matrix differences between the cheeses. Nevertheless, it is important to consider that matrix composition and hydrophobicity of the analytes may have influenced some results. Octanol/water partition coefficient (LogP) values are included in table 3.4 and referenced in the discussions below to highlight where the hydrophobicity of an analyte may have affected its quantitation.

Figure 3.2 PCA plots of semi-quantitative data for 18 cooked Cheddar odorants across six cooked cheeses



Left – variables plot. Right – Observations plot.

Sample abbreviation: Prm – Parmesan ; Mz – Mozzarella ; Chd – Mature Cheddar ; HF – high-fat mild Cheddar ; MF – Medium fat mild Cheddar ; LF – low far mild Cheddar. Volatile abbreviations: 3MB – 3-methylbutanal; 2MB – 2-methylbutanal; DMDS – dimethyl disulfide; BA – butanoic acid; HPT – 2-heptanone; MET – methional; HA – hexanoic acid; DMTS – dimethyl trisulfide; PHE – phenylacetaldehyde; 3MBA – 3-methylbutanoic acid; TMP – trimethylpyrazine; MSH - methanethiol

The concentration of butanoic acid was lower in the low-fat cooked mild Cheddar than in the high-fat mild Cheddar, which is consistent with the relative concentrations in the uncooked cheeses. Although short chain fatty acids can form from triglycerides during cooking (Nawar, 1969), the decrease in concentration of butanoic acid during cooking suggests the majority of butanoic acid in cooked cheese is likely to be residual from the uncooked cheese. Due to the relative hydrophobicity of butanoic acid, it is possible that the difference in concentration of short-chain fatty acids is inflated by the effect of the matrix on their release during headspace extraction.

Table 3.4: Log P values for selected volatiles.

Compound	Log P
methional	0.41 [b]
butanoic acid	0.79 [a]
trimethylpyrazine	0.95 [a]
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	1.03 [a]
3-methylbutanal	1.23 [b]
dimethyl trisulfide	1.87 [b]
hexanoic acid	1.92 [a]
2-heptanone	1.98 [a]
ethyl hexanoate	2.83 [b]

Octanol water partition coefficients (log P) values of selected volatiles from multiple chemical classes. Data obtained from (a) ChemSpider (experimental), (b) ChemSpider (estimated)

Significant ($p < 0.05$) differences were observed in the concentration of Strecker aldehydes between the mild Cheddars of different fat contents. Concentrations of 2-methylpropanal, 3-methylbutanal and methional were all highest in the high-fat

cooked mild Cheddar, while lowest in the low-fat. The difference between low and high-fat was significant ($p < 0.05$) in each case. The Strecker aldehydes are slightly hydrophobic, therefore the matrix differences between the cheeses would be expected to produce the opposite trends to those observed in the data, suggesting that the trends are unlikely to be an artefact of matrix composition.

The concentrations of the corresponding amino acids in the uncooked cheeses (see chapter 4), valine, leucine and methionine were higher the uncooked low-fat mild Cheddar than in the high-fat, although not significantly. This does not correlate with the levels of formation of these Strecker aldehydes in the cooked cheese.

The other reactant in the Strecker degradation mechanism (thermally induced as part of the Maillard reaction) is a carbonyl compound (Strecker, 1862). These compounds are typically formed from the breakdown of reducing sugars. A comparison of reducing sugar concentration in the cheeses (see chapter 4) showed that the concentration of lactose was significantly ($p < 0.05$) higher in the HF than LF cheese. However, the concentration of dicarbonyl may not have been similarly affected, for example 2,3-butanedione was not significantly different in the mild Cheddars of differing fat content. The lower concentration of reducing sugars in the LF cheese is likely to contribute to the lower formation of Strecker aldehydes during cooking.

Additionally, lipid derived reactive carbonyl compounds have also been shown to contribute to the formation of Strecker aldehydes (Hildago & Zamora, 2016; Hildago & Zamora, 2019). The lower concentration of Strecker aldehydes in cooked low-fat mild Cheddar compared to higher fat cheeses suggests that lipid derived carbonyls may be contributing to the formation of Strecker aldehydes in the higher fat mild Cheddars. In the uncooked mild Cheddars there were no significant ($p < 0.05$)

differences between the concentrations of Strecker aldehydes by fat content. This is likely to be because the microbial pathways to their formation in uncooked cheese do not involve dicarbonyls (Ehrlich, 1907), unlike the Maillard reaction. However, in some previous literature methional and phenylacetaldehyde have both been reported as higher in reduced and low-fat uncooked Cheddars than in high-fat Cheddar (Drake, Miracle & McMahon, 2010).

There were few significant ($p < 0.05$) differences between the levels of pyrazines in cooked mild Cheddars of differing fat content, suggesting that fat content does not significantly ($p < 0.05$) affect formation of pyrazines during cooking of cheese. This result is logically consistent with the possible involvement of lipid derived carbonyls in formation of Strecker aldehydes, as the hydroxyl amino compounds produced by that mechanism are not precursors to pyrazines, instead forming 2-alkylpyridines (Hildago & Zamora, 2004). However, alkylpyridines were not detected in any of the cooked cheeses. Only 2,5 dimethylpyrazine was detected in the uncooked mild Cheddars. It was significantly ($p < 0.05$) higher in the high-fat uncooked Cheddar than the medium or low-fat cheeses.

The levels of 2-methylketones detected in the cooked low-fat mild Cheddar were significantly ($p < 0.05$) lower than the other two cooked mild Cheddars. As 2-methylketones are hydrophobic this result is not likely to be caused by differences in flavour release from the matrices of differing fat contents. 2-Methylketones have been shown to form from β-ketoalkanoic acids esterified in the milk fat glycerides via an hydrolysis and decarboxylation reaction upon heating (Nawar, 1969; Calvo and de la Hoz, 1992), so it is a logical result that their concentration was lower in a low-fat cooked cheese than a high-fat cooked cheese. The concentrations of 2-

methylketones in high and medium fat mild Cheddars were similar. The MF cooked Cheddar did not contain significantly ($p < 0.05$) less 2-methylketones than the HF sample, suggesting that a moderate reduction in cheese fat does not significantly ($p < 0.05$) affect 2-methylketone concentration, while a greater reduction has a significant effect. 2-methylketones were also lower in the uncooked low-fat mild Cheddar than in the uncooked high-fat mild Cheddar.

The level of methanethiol and methional increased with fat concentration in cooked mild Cheddars. However, methionine (likely to be the most important precursor to methional formation, although cysteine may also contribute) had a higher concentration in the LF than HF cheese. They are both relatively hydrophilic, so this trend is not likely to be an artefact of matrix release differences. Methanethiol forms from breakdown of methional during the Maillard reaction (Belitz, Grosch & Schieberle 2009), and so the higher concentration of methanethiol in the cooked HF can be attributed to the role of lipids in the formation of Strecker aldehydes (Hildago & Zamora, 2016; Hildago & Zamora, 2019).

Dimethyl disulfide and dimethyl trisulfide are formed from the oxidation of methanethiol. The levels of both dimethyl disulfide and dimethyl trisulfide were highest in the cooked LF, which was correlated with the concentration of methionine, but not correlated with the concentrations of methional or methanethiol. Both of the sulfides are relatively hydrophobic, so the result is unlikely to be an artifact of matrix release differences. The higher concentration of dimethyl disulfide and dimethyl trisulfide in LF cooked Cheddar may indicate that the Maillard reaction had occurred to a greater extent in the cooked LF than in the MF or HF cheeses. The absence of free fat coating low-fat cheeses has been shown to promote rapid dehydration and

browning in low-fat cheeses (Rudan et al, 1999), which are likely to be associated with more extensive Maillard reactions. As dimethyl disulfide and dimethyl trisulfide are formed in later stages of the Maillard degradation of methionine, their high concentration may indicate that these reactions were more advanced in the LF Cheddar.

In the uncooked mild Cheddars, methional, methanethiol and dimethyl disulfide were all similar, while dimethyl trisulfide was significantly ($p < 0.05$) higher in the uncooked high and medium fat Cheddars than in the low-fat Cheddar. This differs with previous data of dimethyl trisulfide concentration in Cheddars of differing fat levels (Drake, Miracle & McMahon, 2010) which found significantly ($p < 0.05$) higher concentrations of dimethyl trisulfide in reduced and low-fat uncooked Cheddar than in high-fat Cheddar.

Cyclotene and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone were both significantly higher in the cooked HF than the cooked LF. Both of these compounds are derived from a sugar moiety in the Maillard reaction or from caramelization, and their higher formation in the HF Cheddar relates to the concentration of sugars detected in the uncooked HF. The sugar concentration in the HF Cheddar was both higher than the LF, and also decreased more during cooking than LF (see chapter 4). 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone is moderately hydrophilic, so it is possible that this result could be influenced by their release from high and low-fat matrices during analysis.

While esters were not detected in any of the cheeses when cooked, there was a positive correlation between fat content in the uncooked mild Cheddars and ester concentration. As ethyl esters are hydrophobic, differences in their release from cheese of differing fat content would be expected to produce the opposite trend, so it

is probable that there are higher concentrations of ethyl esters in the HF compared to LF samples. Formation of esters in cheese can occur via esterification or alcoholysis, although the latter is more prevalent. Alcoholysis occurs from alcohols and fatty acetyl-coenzyme A (Molimard & Spinnler, 1996). A lack of fatty acid precursors in the low-fat cheese may have contributed to their reduced formation, although the alcohol is usually the rate limiting reagent in alcoholysis reaction (Abeijón Mukdsi et al., 2018).

In conclusion, fat content influenced the flavour of cooked Cheddar. 2-Methylketones, fatty acids and Strecker aldehydes had lower concentrations in the lower fat cooked Cheddar. In most cases the trends observed are inconsistent with those expected if all differences were caused by the effect of matrix composition on flavour release during headspace extraction,

Nevertheless further confirmatory studies using a solvent extraction followed by SAFE technique (development of this technique is outlined in chapter 5) were conducted. Results from the SAFE study are presented in chapter 6.

3.4 Conclusion

The odorants in cooked Cheddar have been determined for the first time. When compared to uncooked Cheddar, the aroma of cooked Cheddar is affected by additional compounds including Strecker aldehydes, pyrazines, unsaturated aldehydes, cyclotene and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone . Furthermore, esters such as ethyl butanoate and ethyl hexanoate which have been widely reported as odorants in uncooked Cheddar were not detected by either GC-O or GC-MS in cooked Cheddar despite being present in the uncooked samples. The combination of the additional odorants detected in cooked Cheddar and the loss of odorants from

uncooked Cheddar is likely to affect differences in aroma between uncooked and cooked cheese. Additionally, almost all of the odorants were present at significantly ($p < 0.05$) different levels in one or more cooked cheeses than their uncooked counterparts. This suggests that cooking cheese also affects the balance of odorants, contributing to the change in flavor upon cooking.

Cheese type affected the formation of odorants during cooking, in many cases the formation of volatiles was lower in mozzarella than in the other cheeses. This may be related to the low aging time for mozzarella, as the aging processes such as proteolysis affect the concentration of Maillard reaction precursors. Fat content was also related to the concentration of odorants in cooked mild Cheddar, including the Strecker aldehydes, methanethiol, 2-methylketones and fatty acids. Our results suggest that the fat in cheese is involved with flavour formation during cooking, both directly as a precursor and indirectly due to the role of fat in cheese structure and free fat. Additionally, the lower concentration of dicarbonyls in low-fat cheese may contribute to the lower formation of dicarbonyl derived odorants in low-fat cooked cheese (such as Strecker aldehydes and furanones). These results may have relevance for the dairy industry in creating better performing low-fat cheeses for cooked applications.

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Chapter 4 – Non-volatile characterisation of cooked cheese flavour

Preface to chapter 4

This study explores the contribution of selected non-volatile compounds to cooked cheese flavour. This study tests hypothesis 1, that cooking affects the concentration of umami and kokumi tastants. Furthermore, this work demonstrates the loss of non-volatile precursors to odorant formation, which supports the findings in chapter 3 and 6.

Authors' contributions: As the main author on the study, I completed the material preparation, data collection and data interpretation for the sugars, organic acids and DKP analysis and wrote the first draft of the manuscript. Fiyinfolu Makinwa and Samantha Nottage performed the material preparation and data collection for the γ-glutamyl peptide and amino acid analysis and collaborated with me on the data interpretation. Elements of this study were included in both of their Masters' theses. All authors contributed to the study conception and design and Jane Parker, Colette Fagan and Jose Oruna-Concha provided comments on the draft manuscript.

This chapter has been prepared for submission to journals and will be submitted shortly.

Abstract

This work examined the role of selected non-volatiles in cooked cheese flavour, both as tastants and as precursors to aroma generation in the Maillard reaction. The effect of cooking on the concentration of selected non-volatiles (organic acids, sugars, amino acids, γ-glutamyl dipeptides and diketopiperazines) in six cheeses (mature Cheddar, mozzarella, Parmesan, mild Cheddar (low, medium and high-fat)) was determined. Sugars, amino acids and γ-glutamyl dipeptides decreased in concentration during cooking, while diketopiperazines and some organic acids increased in concentration. Diketopiperazines were above the taste threshold in some cooked cheeses, while below threshold in uncooked cheeses. The role of fat content in cooked cheese flavour is discussed. Furthermore, γ-glutamyl dipeptide concentration increased in concentration during 24 months ageing in low, medium and high-fat Cheddars, with similar levels of γ-glutamyl dipeptides detected in aged low and high-fat Cheddars.

4.1 Introduction

Cheese is a major commodity produced by the dairy industry globally. Mintel (2020) estimate the UK market value for cheese to be £3.2 billion in 2020. Applications for cheese include a variety of cooked dishes such as toppings to pasta and pizza, grilled or melted (e.g fondue). Previous research into cheese flavour has focused on uncooked cheese, and little is known about the effect of cooking on the taste of cheese.

This study aimed to determine the effect of cooking on the concentration of selected non-volatiles in a range of popular cheeses in the UK (Parmesan, mature Cheddar, mozzarella, low-fat mild Cheddar, medium-fat mild Cheddar, high-fat mild

Cheddar). Analytes were selected based on their contribution to taste in uncooked cheese and their potential role as precursors in the Maillard reaction. Five groups of analytes were chosen for analysis: amino acids, sugars, organic acids, γ-glutamyl peptides and diketopiperazines (DKPs).

It is hypothesized that amino acids, peptides and sugars in cheese decrease in concentration during cooking due to participation in the Maillard reaction. Furthermore, some organic acids (e.g acetic acid) and DKPs are hypothesized to increase in concentration due to their formation during cooking. We hypothesise that these changes may be substantial enough to alter the balance of suprathreshold tastants in cooked cheese compared to uncooked cheese.

Organic acids, especially lactic acid are key to the characteristic sharpness and low pH in uncooked cheese (McSweeney, 1997). Amino acids possess various taste properties including bitterness, sweetness, sourness and umami. In particular, glutamic acid has been shown to contribute significantly to umami flavour in cheeses (Fox, 1989; Andersen et al, 2010; McSweeney, 1997). The most abundant sugars in cheese are lactose and its component monosaccharides, glucose and galactose. While these sugars contribute to sweetness in many dairy products, the majority of the lactose in milk is lost to the whey during cheesemaking, so lactose concentrations are below sweet threshold in most cheeses (McSweeney, 1997).

γ-Glutamyl dipeptides have been reported in various uncooked cheeses (Toelstede et al, 2009; Toelstede & Hofmann, 2009; Kilcawley, 2017), where they contribute kokumi taste (mouthfulness). The concentration of γ-glutamyl dipeptides increases during the ageing of gouda (Toelstede et al, 2009; Toelstede & Hofmann, 2009) but their formation in low-fat cheeses during ageing has not been studied. For this reason,

alongside the study of γ-glutamyl dipeptides in cooked and uncooked high, medium and low-fat mild Cheddar, each Cheddar was also ripened to 24 months with regular analysis of γ-glutamyl dipeptide concentration during aging.

Diketopiperazines (DKPs) are cyclic dipeptides that contribute to bitter and metallic flavours (Borthwick & da Costa, 2017). DKPs have been reported in several cooked foods, including beef (M. Z. Chen et al, 2009), chicken essence (Chen et al, 2004), cocoa (Stark and Hofmann, 2005), coffee (Ginz & Engelhardt, 2000), bread (Ryan et al, 2009) and sake (Takahashi et al, 1974). Additionally, they have been reported in uncooked Comté cheese (Roudot-Algaron et al, 1993) but at subthreshold concentration.

Further to the characterization of cooked cheese flavour, this study included a comparison of low and high-fat mild Cheddar during cooking. Fat and calorie reduction is an important focus for the food industry, and certain cheese-containing products such as pizza and cheese-topped ready meals could benefit from the use of lower fat cheeses. Fat can act as a precursor during the Maillard reaction, and also affect the structural and melt properties of cheeses (Guinee et al, 2000; Rudan & Barbano, 1998; Rudan et al, 1999; Mistry, 2001). For this reason, the fat content of cheese has the potential to influence the development of flavour during cooking. The second hypothesis of this study is that fat content influences flavour development during cooking in cheese.

4.2 Materials and methods

4.2.1 Materials

All materials were purchased from Merck (Gillingham, UK) unless otherwise listed below. Dipeptide standards used were; γ-Glu-Glu, γ-Glu-Val, γ-Glu-Met, γ-Glu-Tyr,

ɣ-Glu-Leu and ɣ-Glu-Phe (Merck, Gillingham, UK). DKP standards were; c-Leu-Pro, c-Val-Pro, c-Pro-Pro and c-Ala-Pro (Bachem, Switzerland).

4.2.2 Cheeses

All cheeses used in this study was manufactured using pasteurised milk, except for the Parmesan. Three cheeses were purchased from a supermarket: mature commercial Cheddar (Ched), fresh mozzarella (Mozz) and Parmesan (Parm). Cheddar, mozzarella and Parmesan represent a range of cheeses that are used in cooked dishes and vary considerably in terms of maturity. Three Cheddar cheeses of differing fat content, low-fat (LF, 2 % fat), medium fat (MF, 22 % fat), high-fat (HF, 35 % fat), were made at the University of Reading's pilot plant facility (Reading, UK) as described in 2.3, and were included to determine the effect of fat content on formation of cooked cheese non-volatiles and on formation of amino acids and γ-glutamyl peptides during maturation.

4.2.3 Cheesemaking

As described in chapter 3.

4.2.4 Cheese Sample Preparation

Grated cheese (50 g) was spread evenly on a glass petri dish (90 mm diameter x 10 mm depth) and baked in a GC Oven (Hewlett Packard 5890 Series II) at 180 °C for 20 min. It was then cooled to room temperature, immersed in liquid nitrogen, and ground in a coffee grinder (Quest, Liverpool UK) to obtain a fine powder.

4.2.5 DKP analysis

4.2.5.1 SPE extraction

Cheese (~50 g) was cut into 1 cm^3 pieces, immersed in liquid nitrogen (BOC, UK), and ground in a coffee grinder (Quest, Liverpool UK) to obtain a fine powder. In triplicate, a portion of cheese (uncooked or cooked) (5 ± 0.1 g) was spiked with 50 µL internal standard solution (5-methyl-2-hexanone 0.500 % in isopropyl alcohol) and vortexed vigorously with 25 mL HPLC grade water for 60 min. The slurry was then centrifuged for 3 min at 2038 g and 15 °C. The supernatant underwent solid-phase extraction using SPE cartridges (Strata-X 33 µm polymeric reversed-phase giga tube, Phenomenex). The SPE cartridge was conditioned using 5 mL ethanol followed by 5 mL HPLC grade water. The sample was loaded slowly onto the cartridge and rinsed with a further 5 mL water and then dried by passing air through the cartridge for 30 seconds. The sample was then eluted from the cartridge slowly with 5 mL methyl acetate.

4.2.5.2 GC-MS analysis of DKPs

Analyses were performed on an Agilent 7890-5977A GC-MS system (Agilent, Stockport, UK) equipped with an autosampler (Agilent, Stockport, UK). Each liquid extract (1 µL) was injected in splitless mode onto a DB-FFAP polar column (30 m 0.25 mm I.D., 0.25 µm film thickness), (Phenomenex, Macclesfield, UK). The oven temperature was 45 °C initially, rising by 4 °C/min to 220 °C, and held for 45 min. Helium was used as the carrier gas at 1.2 mL/min. The mass spectrometer was operated in electron ionization mode with a source temperature of 230 °C, an ionising voltage of 70 eV, and a scan range from m/z 40 to m/z 300 at 5.3 scans/s. The data were acquired and analysed using Masshunter software (Version 4.5, Agilent, UK).

Compounds were identified by comparing their mass spectra and linear retention indices with those of authentic standards.

4.2.6 Sugars Analysis

Cheese (~50 g) was cut into 1 cm^3 pieces, immersed in liquid nitrogen (BOC, UK), and ground in a coffee grinder (Quest, Liverpool UK) to obtain a fine powder. In triplicate, a portion of cooked or uncooked cheese (5 ± 0.1 g) was vortexed with sulfuric acid solution (25 ml, 0.09 N) for 1 h. The slurry was centrifuged for 10 min at 2038 g. The supernatant was filtered by gravity (Whatman 1 filter paper) and then through a disk syringe filter (Agilent, 0.45µm pore size, 15mm diameter) then frozen at -20 °C until analysis.

Analysis was performed on an Agilent (UK) 1260 Infinity II LC with Infinitylab XT MSD. Samples (10 µL) were separated through a Ca-phase ion-exchange column (Agilent, UK, 300 x 7.7mm Hi-Plex Ca) heated at 80 °C, at a flow of 0.4 mL/min. The mobile phase was 100 % water (LC-MS grade, Sigma Aldrich). Single-ion-monitoring mode was used for the identification of lactose, glucose and galactose using the sodiated molecular ion (m/z 365, 203 and 203 respectively) alongside a comparison of retention time with authentic standards (Sigma Aldrich). The source fragmentor voltage was 135 V, capillary voltage of 2000 V and nozzle voltage of 1500 V. The gas temperature in the source was 300 °C and the nebuliser pressure was 30 psi. Quantitation was performed by comparison of MS areas against a calibration curve of standard solutions of concentration 0.01 – 100 mg/L (lactose) or 0.01 – 10 mg/L (glucose and galactose) in sulfuric acid (0.09 N).

4.2.6 Organic Acids Analysis

The extracts used for sugars analysis were also used for organic acids analysis. The

organic acids analysed were citric acid, malic acid, lactic acid, acetic acid and propanoic acid. Although some of the acids quantified are volatile, their presence was identified during the analysis of non-volatile acids and they were quantified during the same analysis. Analysis was performed on an Agilent (UK) 1260 Infinity II LC with Infinitylab XT MSD and a diode array UV detector (Agilent, UK). Samples (20 μL) were separated through an H-phase ion-exchange column (Agilent, UK, 300 x 7.7mm Hi-Plex H) heated at 65 °C, at a flow of 0.5 mL/min. The mobile phase was water with 0.01 M sulfuric acid. Each acid was identified by comparison of the retention time and MS with those of authentic standards. The MS operated in both positive and negative scan modes between 50 and 250 m/z, with a fragmentor voltage of 135 V, capillary voltage of 3500 V and nozzle voltage of 2000 V. The gas temperature in the source was 300 °C and the nebuliser pressure was 1.38 bar. The diode array detector operated at wavelengths of 220 and 275 nm. Quantitation was performed by comparison of diode array areas against a calibration curve (5 points, 50-1000 mg/L in the extracts) for each sugar.

4.2.7 Amino Acid Analysis

The method described by Toelstede et al, (2009) was used to prepare a water-soluble extract of each cheese. The uncooked or cooked cheese (12.5 g) was homogenised using a Phillips (Guildford, UK) hand blender for 2 min with deionised water (50 mL). They were centrifuged (Sigma 3k10) for 20 min at 4 °C, and 6654 g. The fat layer was removed and the supernatant was reserved. Deionised water (50 mL) was added to the remaining protein pellet and the samples were shaken for 15 min using a Heidolph multi reax shaker (Heidolph Instruments GmbH & Co, Germany). The samples were centrifuged again under the same conditions as above. The supernatant

was combined with the first supernatant and the pH was adjusted to 4.6 with 98-100 % formic acid. It was then centrifuged and filtered under vacuum. The extracts were freeze-dried and ground into a powder. The freeze-dried samples (200 mg) were rehydrated with 5 mL water and filtered using a 25 mm, 0.2 µm syringe filter. Samples were prepared for amino acid analysis using the EZ: FAAST system (Phenomenex, UK). Analysis was conducted on an Agilent Technologies 6890N GC system coupled to an Agilent 5975 inert XL Mass Selective Detector. The oven was fitted with a ZBAAA GC column. The injection port was held at 250 °C and the oven programme was as follows; 30 °C/min ramp from 110 °C-320 °C. The carrier gas was helium at a constant flow rate of 1.1 mL/min.

4.2.8 γ-glutamyl peptide Analysis

Water soluble extracts were prepared as described in section 2.7. Ɣ-Glutamyl dipeptide (γ-Glu-Glu, γ-Glu-Val, γ-Glu-Met, γ-Glu-Tyr, γ-Glu-Leu and γ-Glu-Phe) analysis was performed according to a modified method to that described by Toelstede and Hofmann (2009). Aliquots (5 µl) of samples were injected into a triple quadruple mass spectrometer (Agilent, Japan) coupled with an Agilent 1260 Infinity HPLC system (Agilent, Japan), fitted with a 2.1 x 100 mm, 1.8 µm ZORBAX SB-C18 column (Agilent, U.S.A.). The mobile phase was comprised of acetonitrile and water, each containing 1 % formic acid. The flow rate was 0.2 ml/min, and the solvent ratio of acetonitrile to water was 0:100 initially, increasing to 10:90 by 10 min and finally increasing to 100:0 by 25 min which was the final runtime. The mass spectrometer was operating in positive EI mode, using the following settings: ion spray voltage 4000 eV, fragmentor voltage 50 eV, collision energy 10, source temperature (TEM) 325 °C and nitrogen curtain gas (CUR) 2.42 bar. Multiple-

reaction monitoring mode (MRM) was performed using the mass transitions previously reported by Toelstede and Hofmannn (2009). Peak areas obtained for corresponding mass traces were compared to those of standard solutions of reference peptides to enable quantitative analysis.

4.2.9 Statistical interpretation

The concentrations of the non-volatile compounds were analysed by one-way analysis of variance (ANOVA) using XLSTAT statistical and data analysis solution (Addinsoft (2020) New York, USA). For those compounds exhibiting the significant difference in the ANOVA, Fisher's least significant difference (LSD) test was applied to determine which sample means differed significantly ($p < 0.05$).

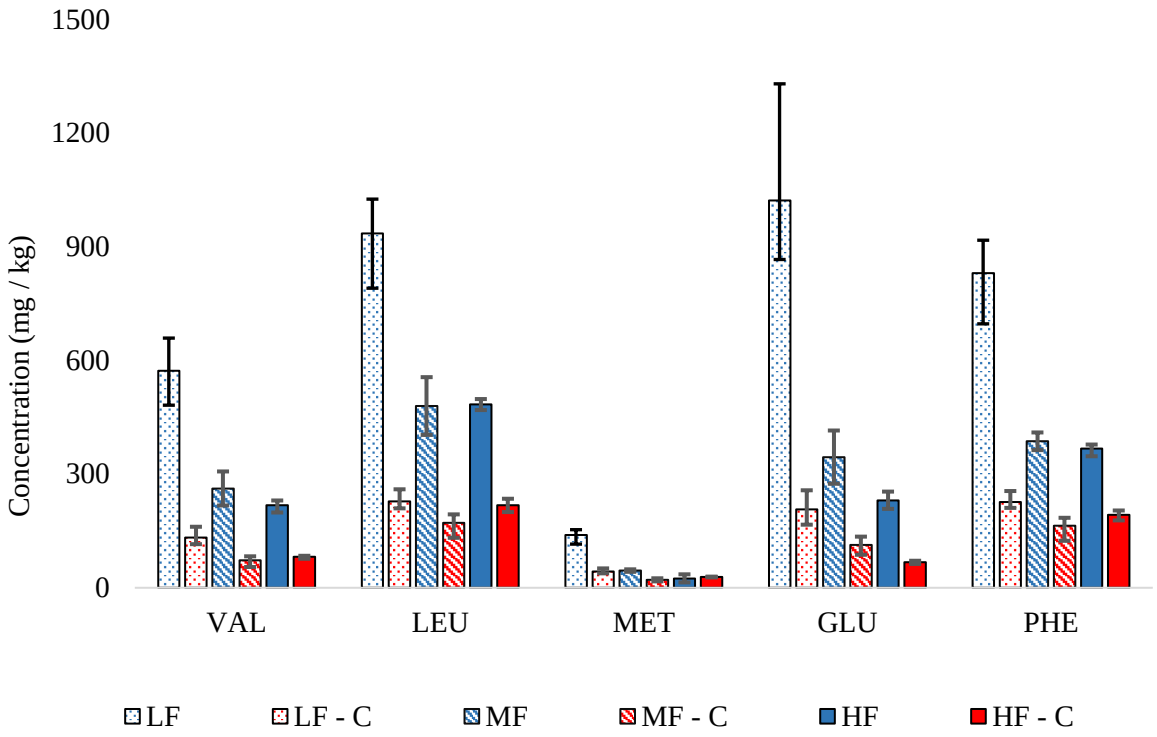
4.3 Results and Discussion

4.3.1 Amino acids

Amino acid concentrations increased during aging in the Cheddars. The low-fat cheeses contained higher concentrations of amino acids than in their MF or HF equivalents, as shown for glutamic acid in figure 4.2. These findings agree with previous studies (Guinee et al, 2000; Altemueller & Rosenburg, 1996), although the rate of proteolysis has also been shown to be typically slower in low-fat cheeses (Rudan et al, 1999; Guinee et al, 2000; McCarthy et al, 2016).

This is related to a lower moisture to protein ratio in low-fat cheeses which negatively affects the ease with which proteolytic microorganisms and enzymes can access their substrates. Amino acid formation occurs during proteolysis, so we attribute higher formation of amino acids in our low-fat cheeses to higher protein concentration, rather than a faster rate of proteolysis.

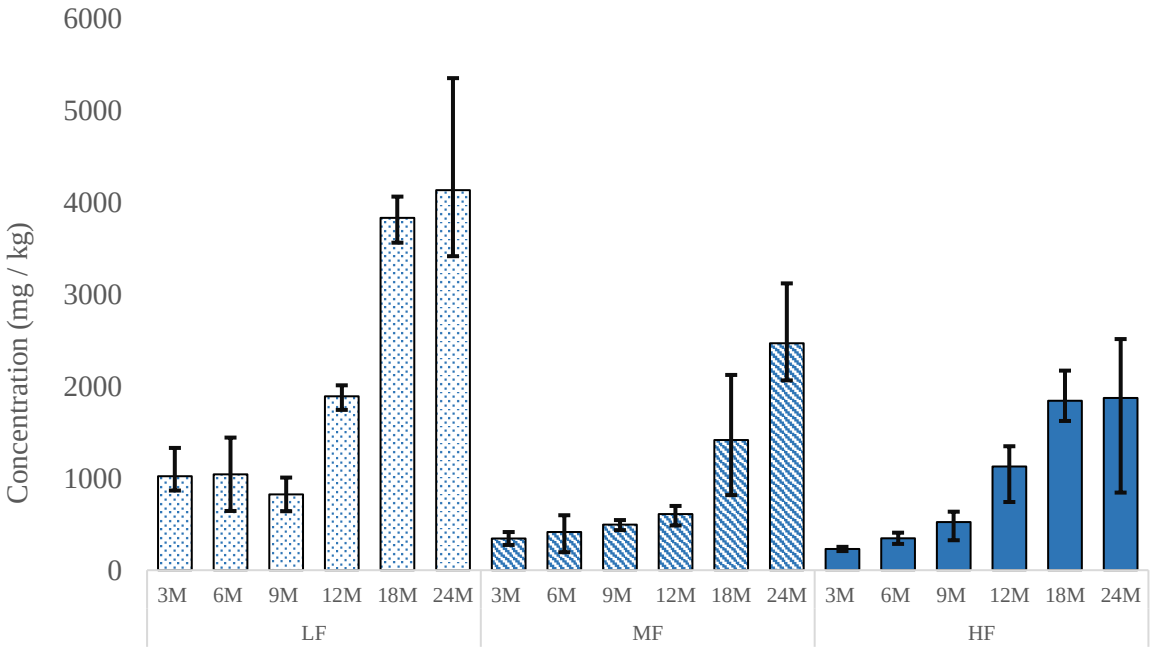
Figure 4.1. Comparison of mean dry weight concentrations of five amino acids in cooked and uncooked Cheddars.



Cheddars are LF (dotted bars), MF (striped bars) and HF (single colour bars). Cooked Cheddars (referred to in key with '-C') are shown with red coloured bars, uncooked Cheddars are shown blue coloured bars. Full amino acid data given in appendix 5.

During cooking, the amino acid concentrations decreased on a dry weight basis in all cheeses. Participation in the Maillard reaction and formation of DKPs is likely to be a major contributor to losses of amino acids during cooking. While the concentration of amino acids was higher in the low-fat cheese, there was also a greater loss of amino acids during cooking in low-fat cheese (72% in LF, compared to 41 and 44% in MF and HF respectively).

Figure 4.2 Mean concentration of glutamic acid in Cheddars throughout ripening.



Ripening periods were 3,6,9,12,18 and 24 months. Data given on a dry weight basis. Patterned bars are LF (spotted bars), MF (striped bars) and HF (solid coloured bars). Error bars on each graph indicate the minimum and maximum range values.

The rapid loss of amino acids during cooking in LF compared to HF may indicate Maillard reactions between amino acids and sugars, however, the loss of sugar from LF was much lower than HF (see section 3.4).

Furthermore chapter 3 shows that greater levels of the volatile Maillard products were lower in the cooked LF than HF Cheddars. It is possible that loss of amino acids in LF cheeses during cooking may occur through a different mechanism to the typical reaction with reducing sugars in the Maillard reaction.

Alternatively, another possible explanation for this difference is the physical effect of fat on the cooking process in cheese. Cheese structure is an amorphous casein network interspersed with globules of fat, moisture and other components. During cooking, the fat globules coalesce and eventually pool into a free fat layer which coats the cheese. In low-fat cheeses, there are fewer and smaller fat globules to interrupt the protein phase (McCarthy, 2016), and a lower moisture to protein ratio. This more continuous casein network may provide more opportunity for the thermally induced reactions involving amino acids to occur. Furthermore, in low-fat cheeses, it has been shown that the absence of a free fat layer promotes rapid dehydration and browning (Guinee et al, 2000; Rudan & Barbano, 1998; Rudan et al, 1999), indicating the occurrence of thermally induced reactions.

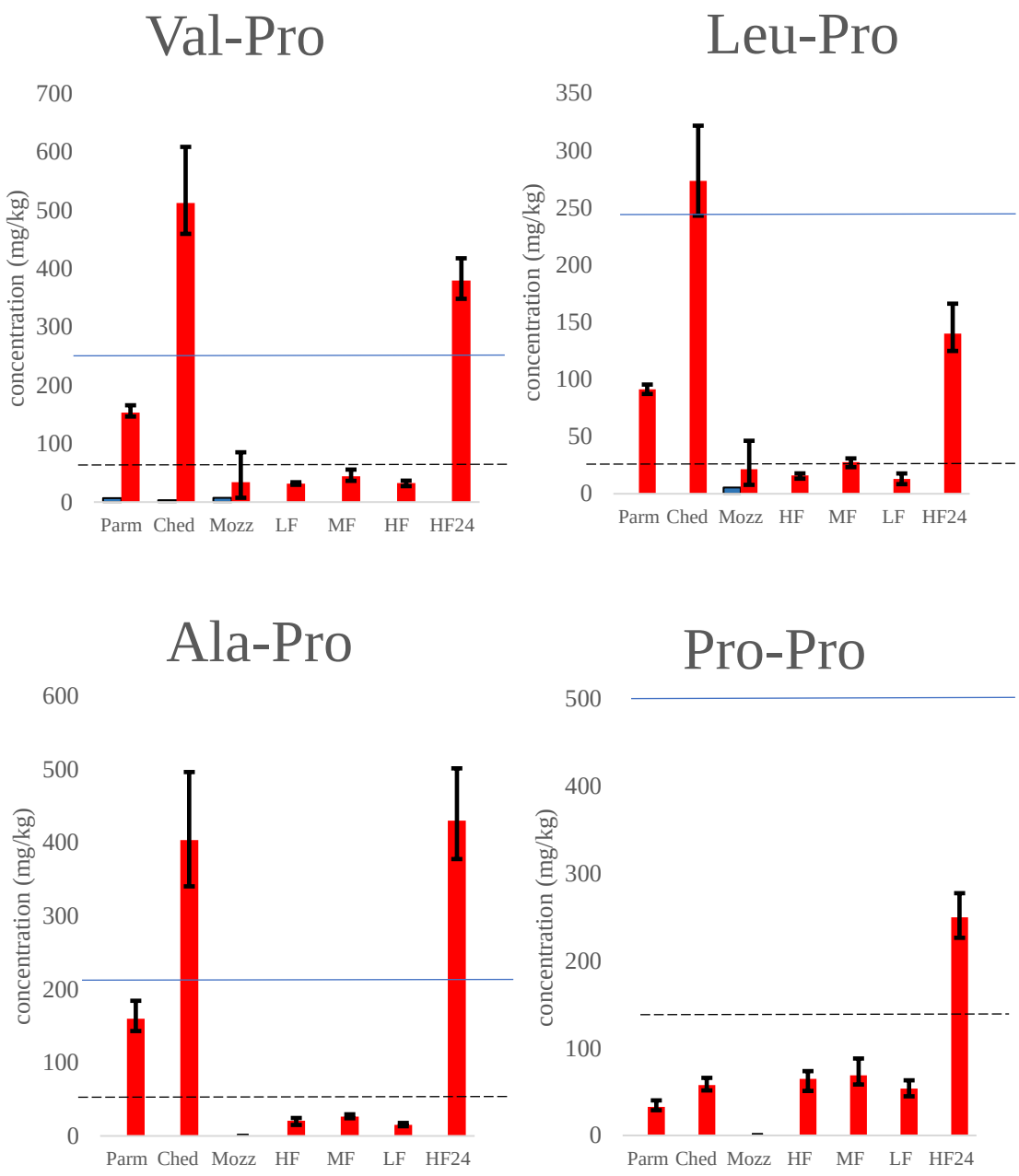
The concentration of most amino acids was below their taste thresholds (Hillmann & Hofmann, 2016) in the mild Cheddars, however isoleucine, aspartic acid and glutamic acid were all above threshold in uncooked LF, and glutamic acid was also above threshold in uncooked MF and HF. In the cooked cheeses, glutamic acid was above threshold in LF only. This suggests that glutamic acid may contribute to umami flavour in some cooked cheeses, although glutamic acid concentration decreased substantially during cooking.

4.3.2 DKPs

Figure 4.3 shows the concentration of four DKPs detected in the cooked and uncooked cheeses on a wet weight basis, along with their metallic and bitter taste thresholds (Stark and Hofmann, 2005). All DKPs detected were subthreshold in the uncooked cheeses. This agrees with a previous study, which showed that DKPs are present at subthreshold concentrations in uncooked comté and do not contribute to

bitter taste (Roudot-Algaron et al, 1993).

Figure 4.3 Mean concentration of four proline-containing DKPs.



Data are given on a wet weight basis. Cheeses were cooked (red bars) and uncooked (blue bars). Black dashed line - Metallic taste threshold. Blue solid line - Bitter taste threshold (Stark & Hofmann, 2005). Error bars show minimum and maximum range values. N=3. Data shown in appendix 4.

2548 However, the concentrations of DKPs increased significantly ($p < 0.05$) (5 to 150
2549 fold higher) during cooking. Furthermore, some DKPs were detected in the cooked
2550 cheeses which were not detected in their uncooked counterparts. Multiple DKPs were
2551 present above their bitter taste thresholds in the cooked Ched and HF24 samples, and
2552 above their metallic thresholds in Parm.

2553 Regarding the role of fat concentration, the DKPs were all sub-bitter threshold in the
2554 three mild Cheddars, although c-Leu-Pro was above the metallic threshold in MF.
2555 There were no significant differences in DKP concentration between the HF, MF and
2556 LF Cheddars and no trends were observed between fat content and DKP formation.

2557 The observation that the Ched (commercially purchased mature Cheddar) contained
2558 significantly ($p < 0.05$) more DKPs when cooked than any of the mild Cheddars in
2559 the study, suggested that the ageing period of Cheddar may be correlated with DKP
2560 formation when cooked.

2561 Uncooked and cooked samples of the HF Cheddar matured to 24 months were also
2562 tested for DKP concentration. DKPs were significantly ($p < 0.05$) higher in the
2563 cooked HF24 than in HF and were similar in concentration to those detected in
2564 cooked Ched.

2565 As DKPs were not detected in uncooked HF24, this suggests that an increased ageing
2566 period led to the formation of precursors to DKP formation in uncooked Cheddar.
2567 DKP formation occurs from small peptides during thermal processing (Borthwick
2568 and Da Costa, 2017). Proline-containing-DKPs form from di-and-tripeptides with
2569 proline in the second position from the N-terminal (Otsuka et al, 2019).

2570 The formation of DKPs from di-and-tripeptides has been shown to occur both in the
2571 presence and absence of glucose (Lu et al, 2005). The process of proteolysis during

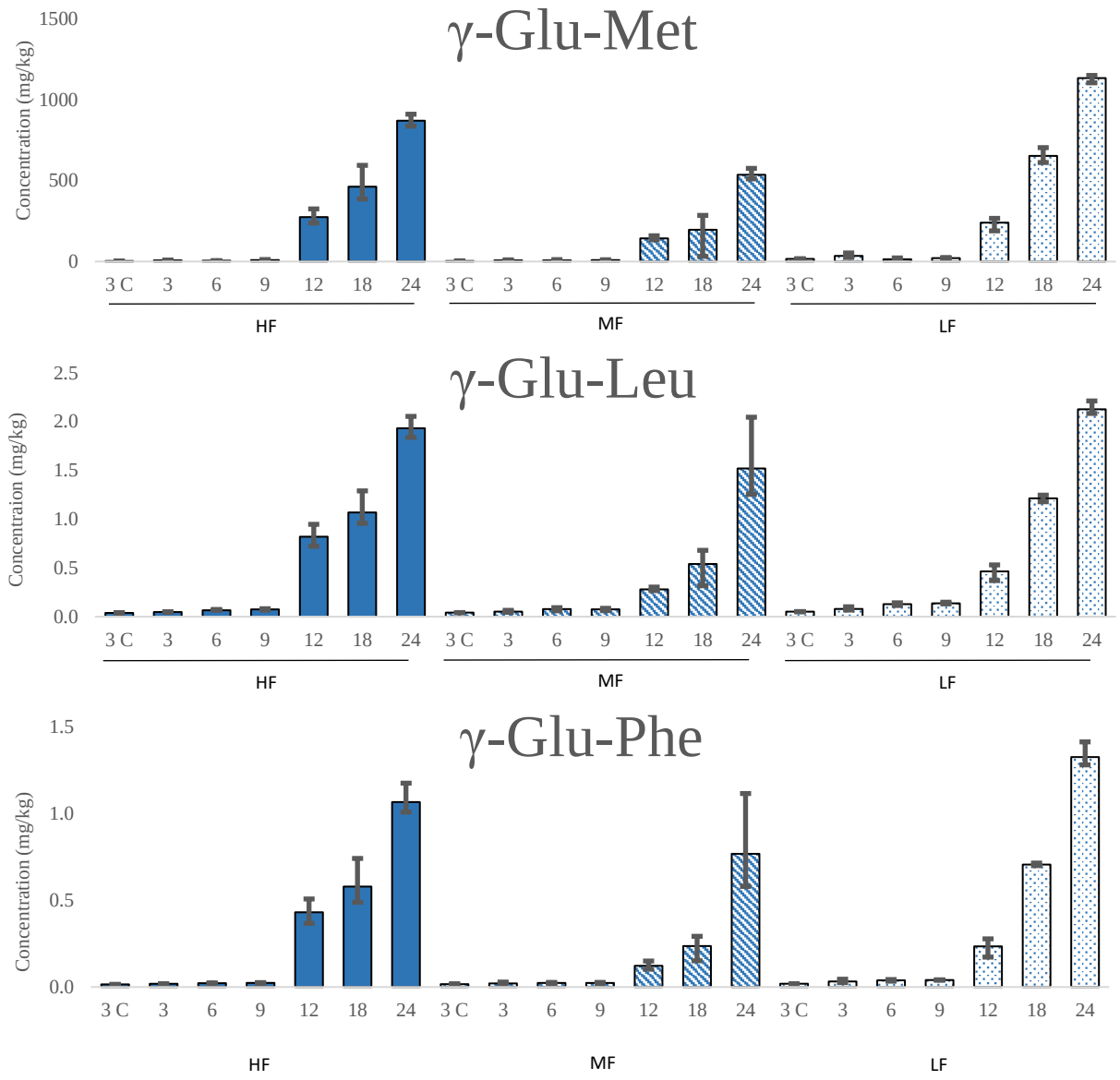
cheese ripening generates small chain peptides and amino acids from cheese proteins (Murtaza et al, 2014). It is likely that the formation of peptides which are precursors to DKPs occurs during Cheddar ripening.

4.3.3 γ-glutamyl peptides

Each of the γ-glutamyl peptides studied (γ-Glu-Glu, γ-Glu-Val, γ-Glu-Met, γ-Glu-Tyr, γ-Glu-Leu, γ-Glu-Phe) increased in concentration during ageing, three examples are shown in figure 4.4. This confirms previous work by Toelstede and Hofmann (2009), which showed a higher concentration of kokumi peptides in 44-month aged gouda than 4 months aged. These changes are driven by the process of proteolysis during cheese ageing, in which long-chain proteins are broken down into smaller chain peptides. The highest concentration was γ-Glu-Met, which also had the largest increase in concentration during ageing. The concentration of γ-Glu-Met at 12 months aged HF Cheddar was comparable to values reported by Toelstede and Hofmann (2009) for ripened goats cheese and higher than values reported for 30 weeks aged Milner and 8 months aged Gruyère, suggesting that Glu-Met formation during ripening progresses at a comparable speed in HF Cheddar to these cheeses.

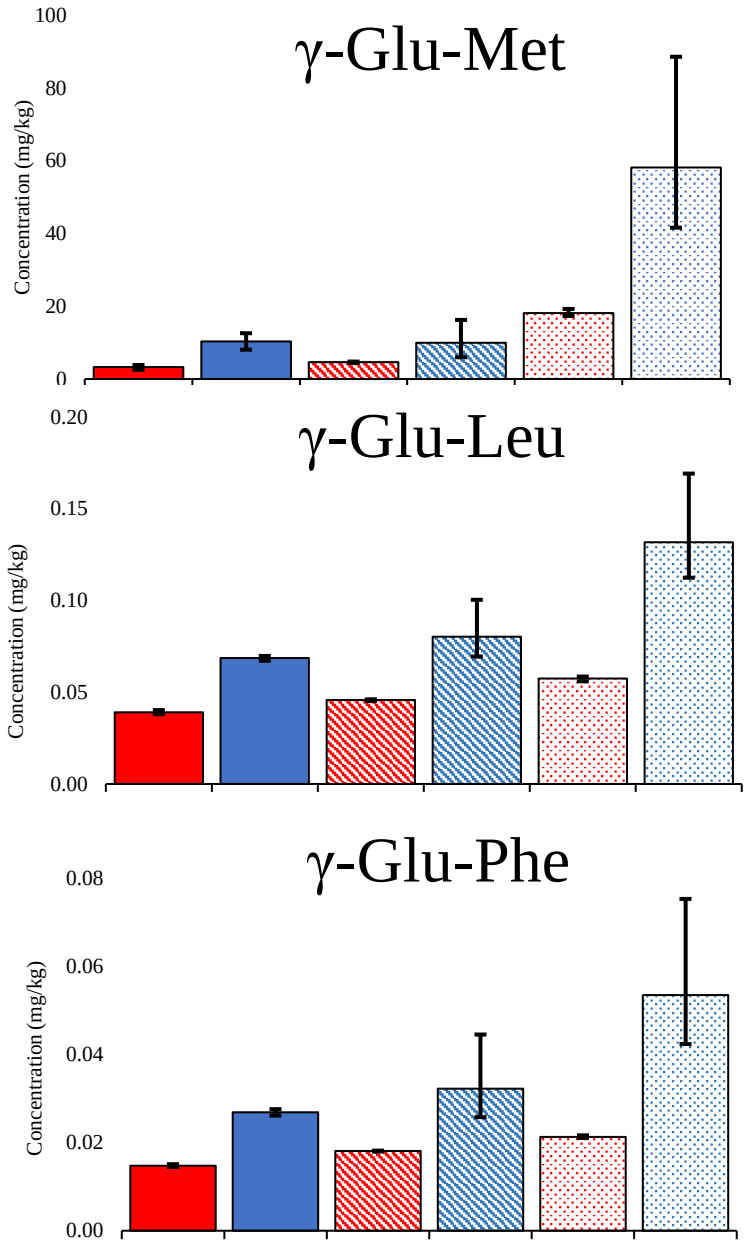
However, the highest concentration of γ-Glu-Met reported (1136 mg/kg in 24 months aged LF Cheddar) was higher than the highest concentration reported by Toelstede and Hofmann (2009) in blue Shropshire. This high value is likely to be driven by the extended ageing period used during our study. The other γ-glutamyl peptides had lower concentrations than some aged cheeses reported in other studies. This difference is likely to be related to the different cultures used in the manufacture of the various cheeses.

Figure 4.4 Mean concentration of three γ-glutamyl peptides throughout ripening.



Ripening period was 3,6,9,12,18 and 24 months respectively. Patterened bars are LF (spotted bars), MF (striped bars) and HF (solid coloured bars). 3C was cooked 3 month aged Cheddar. Data given on a wet-weight basis. Error bars indicate the minimum and maximum range values.

Figure 4.5 Mean concentration of three γ-glutamyl peptides in cooked and uncooked Cheddars.



Cheddars were HF (solid colour bars), MF (striped bars) and LF (spotted bars), uncooked (blue bars) and cooked (red bars). Data given on a dry weight basis. Error bars indicate the minimum and maximum range values.

The concentration of γ-glutamyl peptides in the 24-month aged cheeses was not significantly ($p < 0.05$) different between the LF and HF samples. However, in the uncooked mild Cheddars (3 months aged) only three γ-glutamyl peptides were detected (γ-Glu-Met, γ-Glu-Leu and γ-Glu-Phe). In each case, there were significantly ($p < 0.05$) more γ-glutamyl peptides in the LF mild Cheddar than in the HF. This suggests that higher concentrations of γ-glutamyl peptides are generated during cheesemaking and the early stages of ripening in low-fat Cheddar, but after more extensive ripening the concentration is independent of the fat level in the cheese. The initial high concentration in LF cheese may be related to the higher protein concentration, while the subsequent more rapid formation of γ-glutamyl-peptides in the HF cheese may be reflective of a higher rate of proteolysis. These results indicate that overall kokumi character is likely to be similar in aged HF and LF cheeses alike.

Threshold values (in a WSE from cheese) for γ-Glu-Met, γ-Glu-Glu and γ-Glu-Leu were reported by Toelstede et al (2009). Comparison of these values with the concentrations in our Cheddar demonstrates that γ-Glu-Met and γ-Glu-Leu are present above their thresholds in 24 months aged Cheddar, and Glu-Met is above its threshold uncooked and cooked 3 month aged Cheddars. Thresholds of these peptides in a cheese matrix have not yet been calculated, however, these data confirms previous studies that suggest it is likely that γ-glutamyl peptides play a role in aged cheese flavour. Furthermore, this study indicates that LF Cheddar generates γ-glutamyl peptides at a similar rate to normal Cheddar.

In the cooked mild cheeses, the γ-glutamyl peptides were lower on a wet weight basis than their uncooked counterparts. Furthermore, comparison on a dry weight basis (to

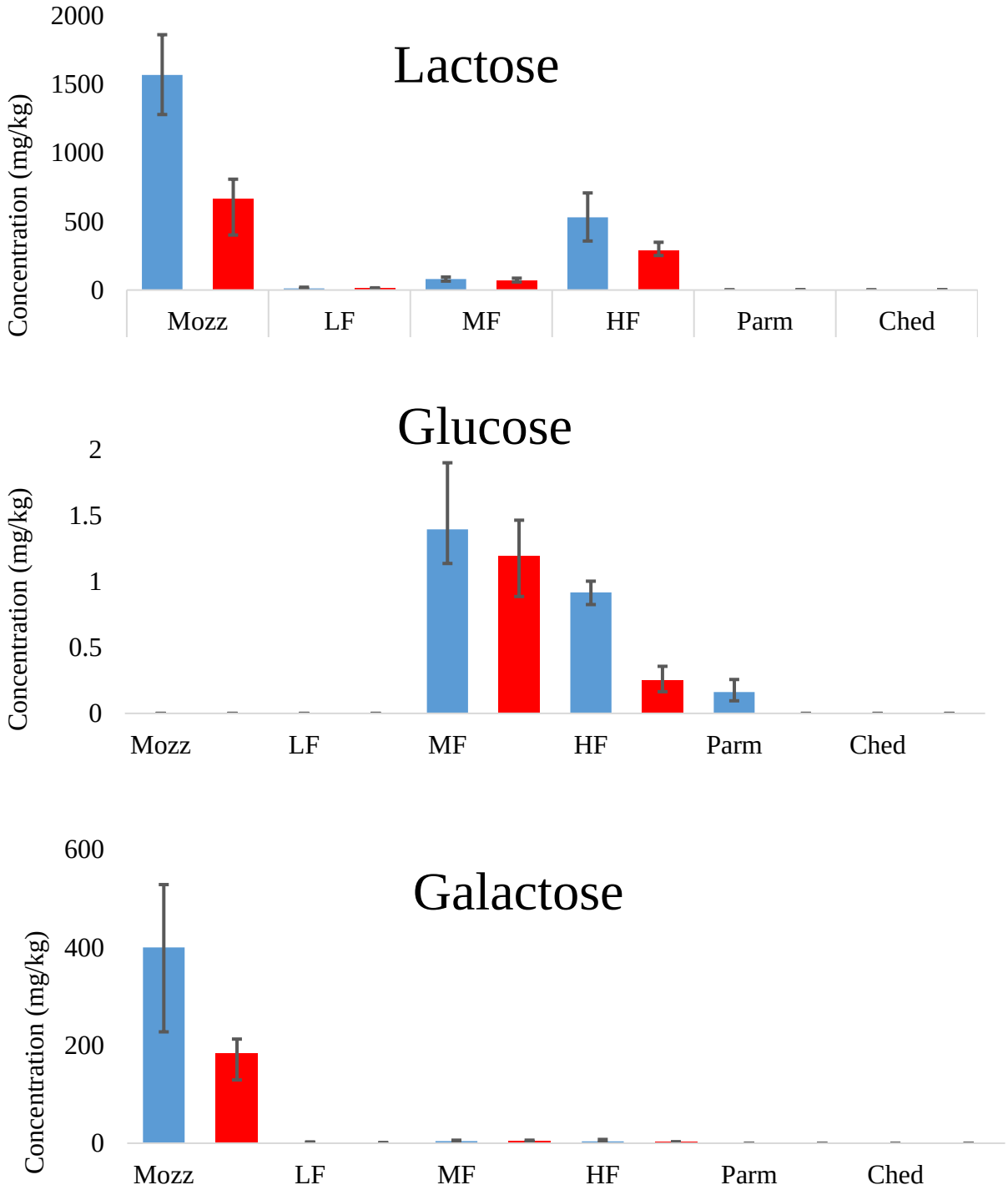
account for the loss of moisture in the cheese during cooking) indicates substantial losses of γ-glutamyl peptides during cooking (43- 69 %, see figure 4.5). The concentrations were highest in the LF uncooked cheese but were more similar across the cooked cheeses. Previous literature has shown that dipeptides are susceptible to the Maillard reaction and can act as precursors to volatile compounds such as pyrazines (van Lancker et al, 2010). While the concentration of γ-glutamyl dipeptides decreased during cooking, γ-Glu-Met was the only one above its threshold in the uncooked WSE and was still above the threshold in the cooked WSE.

4.3.4 Sugars

In the full-fat uncooked cheeses, the concentration of sugars was inversely related to the typical aging period (McSweeney, 2017) of each cheese (mozzarella > mild Cheddar > mature Cheddar and Parmesan) . Figure 4.6 show the concentration of lactose, glucose and galactose in each cheese, both uncooked and cooked, on a dry weight basis. Lactose metabolism occurs during the early stages of cheese ripening and significantly decreases lactose concentration between fresh and aged cheeses (McSweeney et al, 2017). The metabolism of lactose generates its two monosaccharide components, glucose and galactose. Much more galactose was detected in all samples than glucose, similar results have previously been reported (Upreti et al, 2006). When lactose is cleaved the glucose moiety becomes a reactive leaving group which is prone to undergo further reactions, while the galactose produced is relatively less reactive. This is likely to contribute to the difference in concentration between glucose and galactose in uncooked cheeses.

In the cooked cheeses similar concentrations of each of the sugars were detected compared to their uncooked counterparts (on a wet weight basis, see appendix 6).

Figure 4.6 Mean dry weight concentrations of sugars in the six cheeses, cooked and uncooked.



Uncooked (blue bars) and cooked (red bars). Error bars show minimum and maximum range values. Tabular data are shown in appendix 6.

The concentration of lactose in each case was far below the threshold value (Fabian, 1945), suggesting that lactose and the other sugars don't impart sweetness in mild cooked or uncooked Cheddar. This is expected, as the sweetness in cheese is more often attributed to amino acids (e.g threonine, serine, glycine, alanine) and salts (calcium and magnesium propanoates) (Niimi et al, 2014). However, these sugars are involved in the development of flavour during the cooking of cheese as they can act as precursors to other non-volatile and volatile compounds through the Maillard reaction and caramelization mechanisms. Comparison of sugar concentration on a dry weight basis (to account for moisture loss during cooking) demonstrates that as much as 58% of the sugars were lost during cooking.

The sugar concentration in the uncooked mild Cheddars decreased significantly ($p < 0.05$) in the order HF > MF > LF. A positive correlation between lactose concentration and fat content in cheese has been reported previously (McCarthy et al, 2015; 2016). It is related to the rate of lactose metabolism during the cheesemaking and ageing process, as low-fat cheeses are prone to more rapid lactose metabolism. It may also indicate that the starter culture did not sifficiently metabolise lactose. The difference in galactose composition between LF, MF and HF uncooked cheeses was not significant, indicating that further reactions of galactose may also happen more rapidly in low-fat Cheddar. The difference in lactose concentration (dry weight basis) between uncooked and cooked samples was also larger in the HF cheese, suggesting that more sugars are involved in Maillard or caramelisation reactions during cooking in high-fat cheese.

4.3.5 Organic acids

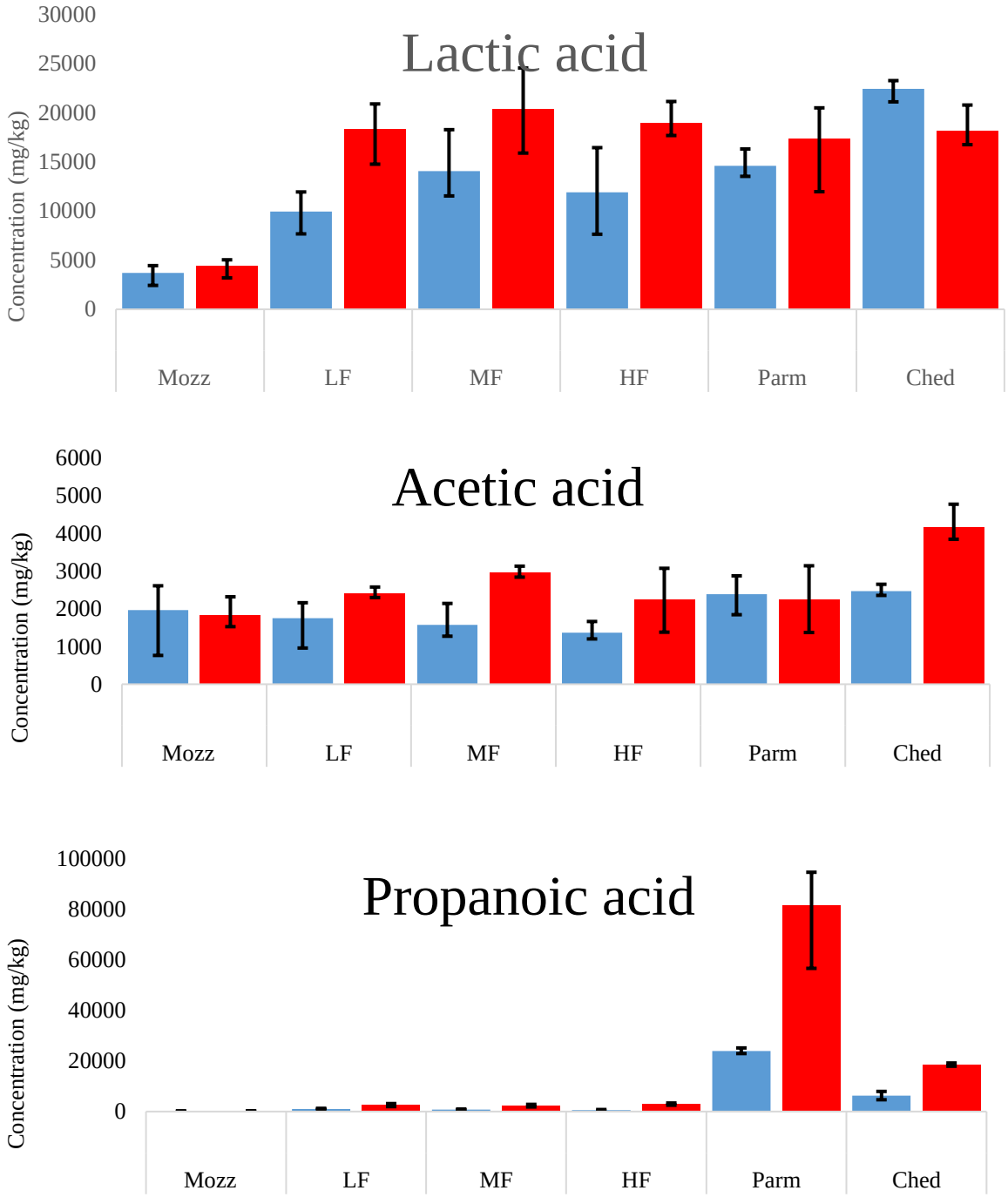
Figure 4.7 shows dry weight comparisons of lactic, acetic and propanoic acid in

uncooked and cooked cheeses. All samples were substantially above the acetic acid and lactic acid threshold for acidic taste in water (Pangborn, 1963). In the uncooked cheeses, the lactic and propanoic acid concentrations were lowest in mozzarella. This may be due to mozzarella being a fresh (un-matured) cheese, as organic acid formation occurs during cheesemaking and maturation. Additionally, the high moisture content of mozzarella compared to the other cheeses studied has a diluting effect on the wet weight concentration of organic acids, as the dry weight concentrations in mozzarella were much closer to the other cheeses.

The propanoic acid concentrations in the uncooked cheeses increased with the typical length of maturation (mozzarella < mild Cheddars < mature Cheddar < Parmesan). Additionally, it is likely to be highest in parmesan due to the inclusion of propionibacteria in the starter cultures. The lactic acid concentrations were also higher in the more aged cheeses, but there was less difference in the lactic acid concentrations than in the propanoic acid concentrations. Similar results have been reported previously (Akalin et al, 2002). McSweeney et al (2017) summarise how most residual lactose is metabolised rapidly after cheesemaking, such that lactic acid concentrations only increase marginally with longer maturation periods. This agrees with the results discussed in section 3.4, in which the highest sugar concentrations were found in the youngest cheeses. In contrast, propanoic and butanoic acids are formed initially by the metabolism of lactose (McSweeney et al, 2017), by lipolytic processes which continue throughout the aging period (Akalin et al, 2002) and also via amino acid catabolism (Banks et al, 2001).

During cooking, the acids increased in concentration on a wet weight basis. On a

Figure 4.7 Mean dry weight concentrations of three acids across six cheeses, cooked and uncooked.



Cheeses are uncooked (blue bars) and cooked (red bars). Error bars show minimum and maximum range values.

dry weight basis, the concentration of acetic acid was similar in the uncooked and cooked cheeses, while the lactic and propanoic acids increased in concentration. The conversion of sugars into small chain organic acids during cooking occurs during the Maillard reaction (Davidek et al., 2006). However, the increase in the concentration of organic acids is higher on a molar basis than the loss of sugars (section 4.3.4).

4.4 Conclusion and implications for cooked cheese flavour

This study has demonstrated that there are changes in the concentration of selected non-volatiles in cheese during cooking. In some cases, the change in concentration during cooking altered which tastants were suprathreshold, which is likely to contribute to differences in flavour between uncooked and cooked cheese.

Sugars, amino acids and γ-glutamyl dipeptides all decreased in concentration, which is likely to be due to their participation in the thermally induced reactions such as the Maillard reaction. While the concentration of γ-glutamyl dipeptides decreased during cooking, their concentration is highly dependent on the extent of maturation of the cheese. Cooking caused up to 69 % loss of γ-glutamyl dipeptides, while ageing from 3 to 24 months resulted in an over 120 fold increase. Cooked aged cheeses may therefore possess kokumi character due to these dipeptides. As with uncooked cheese, sugars are below their taste thresholds in cooked cheeses and unlikely to contribute directly to the flavour. Glutamic acid was suprathreshold in some uncooked cheeses, but subthreshold in some of their cooked counterparts, suggesting that cooking may decrease the umami character of cheeses.

We report for the first time that DKPs increased in concentration during cooking in cheese, and were above taste thresholds in some cooked cheeses. This suggests that bitterness may contribute more substantially to cooked cheese flavour than to

uncooked cheese flavour, especially in cooked mature cheeses. Lactic and propanoic acids concentrations increased during cooking and were substantially above the acidic threshold in all samples both cooked and uncooked. Acidic taste is likely to be as important to cooked cheese flavour as it is to uncooked cheese. Both DKP and organic acid formation are likely to be due to the thermally induced reactions.

Our results suggest that fat may influence flavour formation during cooking in cheese. The loss of amino acids was more rapid in LF than HF Cheddar, although the loss of sugars was more rapid in the HF than LF cheese. Rapid dehydration and browning during cooking has been shown to occur in low-fat cheeses. This is attributed to the lack of a free fat layer coating low-fat cheese compared to regular fat cheeses during cooking.

In addition to the implications for the taste of cooked cheese, changes in the concentration of selected non-volatiles (losses of amino acids, peptides and sugars) in cheese may have implications for the formation of volatiles during cooking, through thermally induced reactions including the Maillard reaction and caramelisation, as discussed in chapter 3.

An explanation for the higher losses of sugars in HF than LF during cooking could be caramelization reactions in HF, which contained over 20 fold more lactose than LF. The volatiles 3- methyl -1,2-cyclopentanedione and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone are both possible products of caramelization, and were 10 fold higher in the cooked HF than LF (chapter 3) although their formation can also occur through Maillard reaction pathways.

This chapter has explored the role of cooking in key non-volatiles in cheese. Along with chapter 3, it is hoped this will give valuable insight for the dairy industry to

inform development of cheeses, especially low-fat variants, for use in cooked foods.

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Chapter 5 - Dilution of solvent extracts from cheese improves yield of higher boiling point volatiles during solvent assisted flavour evaporation

Preface to chapter 5

Comparison of high-fat and low-fat cheese was a key objective from this study and so a suitable extraction method for analysis of high-fat cheese and comparison with low-fat cheese was required. As described in the literature review, SAFE is typically used for this purpose. During the process of validating a SAFE approach for obtaining cheese extracts, a new approach involving dilution of the extracts to a low fat concentration before SAFE was developed. As the comparison on the yield of volatiles during SAFE in low and medium fat solvent extracts was novel data, the study was accepted for publication.

Authors' contributions: As the main author, I conducted the material preparation, data collection, data analysis and wrote the manuscript. All authors contributed to the conception and design and provided comments on the manuscript.

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Abstract

Previously published data show that high levels of fat (50%) affect the yield of volatile compounds during solvent assisted flavour evaporation (SAFE). We present new data demonstrating that even low levels of fat (<10%) lead to significantly ($p < 0.05$) lower yields of high boiling point volatiles during SAFE. Relative recovery during SAFE of a range of volatiles from a cheese extract was measured at varying fat concentrations (1.1–8.7%) using a single internal standard. Volatiles with higher boiling points had significantly ($p < 0.05$) lower relative recoveries, and volatiles were substantially less well recovered from higher fat extracts. When endeavoring to obtain solvent extracts of fatty foods for the purposes of GC-O, it is important to choose the extraction technique which produces solvent extracts closely representing the true composition of the food. We present dilution of solvent extracts prior to SAFE as a potential new approach for high-fat foods which enables high yields of volatiles regardless of boiling point. These data also show that in the absence of C13 labelled standards for quantitation, it is critical to maintain a consistent fat content between samples during SAFE.

5.1 Introduction

Solvent assisted flavour evaporation (SAFE) is a widely used technique for the removal of fat extracted from foods prior to gas chromatographic analysis. Removal of fat is an essential step in producing solvent extracts from high fat foods, as the quantity of lipid material is usually significant and, if not removed, causes issues with the concentration of solvent extracts and the contamination of chromatographic equipment. Previous work (Engel, Bahr & Schieberle, 1999; Lewis Jones, personal communication) has indicated that high fat content affects the relative recovery of

volatile compounds during SAFE. Engel et al. (1999) used a 50% diethyl ether dilution of triacylglycerides as a model organic extract containing a high level of a model fat. Their work demonstrated that SAFE is less effective at recovering volatile compounds when high concentrations of fat are present in the organic extract. However, Engel et al. did not investigate the effect of moderate fat levels on the efficacy of SAFE. This topic is of relevance for further investigation as solvent extracts from high-fat foods often contain moderate levels of fat when undergoing SAFE. This is of particular importance in studies focusing on aroma differences between low and high-fat versions of a food. One such food which has been compared in low and high-fat versions is cheese. The hypothesis of this work was that even moderate to low levels of cheese fat in a solvent extract would affect volatile yields during SAFE.

Cheese aroma is typically studied by extraction of volatile flavour compounds from the cheese matrix, followed by identification and quantification using gas chromatography mass spectrometry (GC-MS). Odorants in cheese can be identified using gas chromatography olfactometry (GC-O) and related techniques such as aroma extract dilution analysis (AEDA). Choice of extraction technique is key to this process since it can have a significant impact on both the quality and quantity of the compounds identified.

Extraction techniques for volatile compounds can be divided into two classes: solvent extraction techniques and headspace techniques. Petersen, Tammam & Ardö (2006) previously studied the effect of fat content of cheese on extraction efficiency during dynamic headspace extraction. They found significant differences between the recovery of some volatiles from cheeses of varying fat content, which was attributed

to the hydrophobicity of the compounds. To facilitate repeat analysis and avoid selectivity based on volatility, solvent extraction is often preferred over headspace extraction techniques.

The focus of this work was on solvent extracts containing cheese fat, however, it is likely that findings will be more widely applicable to extracts from other high fat foods.

5.2. Materials and methods

5.2.1. Reagents and chemicals

Aroma chemicals and the internal standard solution (0.500 % 5-methyl-2-hexanone in isopropyl alcohol) were all obtained at >99% purity from Synergy (High Wycombe, UK). Diethyl ether was obtained from Sigma-Aldrich Ltd. (Gillingham, UK).

5.2.2. Design of analyte mixture and internal standard

To evaluate the efficacy of SAFE across a range of different volatiles, an analyte mixture containing compounds of varying functional group, volatility and hydrophobicity was designed. In preliminary work, an extract of the cheese without spiked analytes was analysed by GC-MS to confirm that none of the selected analytes were present in the cheese itself, nor were any of the analytes likely to coelute with compound peaks from the cheese. The analyte mixture consisted of each of the compounds displayed in Table 5.1 (Group A) at 0.5 % concentration in isopropyl alcohol.

Table 5.1. Boiling points and octanol water partition coefficients (log P values) of Group A (volatiles chosen for analyte mixture) and Group B (volatiles used by Engel et al. (1999))

Compounds	Code	Boiling points (°C)	Log P
Group A			
ethyl butanoate	EB	120[a]	1.85[c]
hexanal	HX	130[a]	1.78[c]
2,5-dimethylpyrazine	DMP	156[a]	0.63[c]
dimethyl trisulfide	DMTS	183[a]	1.87[d]
limonene	LM	176[a]	4.57[c]
4-anisaldehyde	4AA	248[a]	1.76[c]
γ-decalactone	GDL	281[a]	2.72[c]
vanillin	VAN	285[a]	1.37[c]
raspberry ketone	RK	292[b]	0.76[c]
5-methyl-2-hexanone (internal standard)		144[a]	1.88[c]
Group B			
3-methylbutanoic acid	3MBA	176[a]	1.16[c]
phenylacetaldehyde	PAC	195[a]	1.78[c]
2-phenylethanol	PEA	218[a]	1.36[c]
(E,E)-2,4-decadienal	DD	248[a]	
(E)-β-damascenone	BDAM	274[a]	3.41[b]
vanillin	VAN	285[a]	1.37[c]
3-hydroxy-4,5-dimethyl-2(5H)-furanone	SOT	312[b]	1.03[b]

Data obtained from (a) Scifinder (experimental), (b) ChemSpider (experimental), (c) PubChem (experimental), (d) ChemSpider (estimated)

One internal standard (5-methyl-2-hexanone) was added to correct for instrumental drift and minor losses of solvent during SAFE. The internal standard was added to the powdered cheese along with the analyte mixture. It was chosen to have a reasonably low boiling point to maximise recovery during SAFE and make it comparable to the lower boiling point analytes chosen for the study.

5.2.3. Cheese extract preparation

The cheese used during this study was medium Cheddar containing 35 % fat, purchased from Tesco (High Wycombe, UK) on the day of analysis and stored at 4 °C before use. Cheese (~200 g) was cut into 1 cm^3 pieces and frozen rapidly in liquid nitrogen prior to blending in an electric blade-based coffee grinder (Sonifer, Amazon, UK) for 30 s. In triplicate, a portion of cheese (50 ± 1 g) was spiked with 200 µL analyte mixture and 200 µL internal standard solution (5-methyl-2-hexanone 0.500 % in isopropyl alcohol), left to equilibrate for 5 min and extracted using 200 ml diethyl ether by stirring for 1 h. The remaining cheese solids were allowed to settle from the extract and removed by paper filtration and pressed within a filter paper to minimise loss of the extract. A portion of the resulting extract evaporated to dryness confirmed the fat content to be 8.7%. After extraction, four aliquots of 20 ml were separated and diluted with diethyl ether respectively to 8.7, 4.4, 2.2 and 1.1 % fat. Each extract underwent SAFE and the process was carried out in triplicate. Extracts were analysed by GC-MS before and after SAFE, the pre-SAFE samples containing fat were injected last, as they caused significant dirtying of the chromatographic system.

The analyte mixture and internal standard solution (200 µL each) were also spiked directly into 200 ml diethyl ether producing a "0 % fat dilution" which underwent SAFE in triplicate and was analysed by GC-MS before and after SAFE.

5.2.4. SAFE extraction

Samples underwent SAFE extraction using glassware conforming to that described in previous literature (Engel et al, 1999). The water bath and circulatory water were heated to 40 °C and the cooled flask was submerged in liquid nitrogen. The samples were added dropwise such that consistently low pressure (6–9 x 10^{-4} kPa) was maintained.

5.2.5. GC-MS analysis of volatile compounds

All volatile analyses were performed on an Agilent 7890-5977A GC-MS system (Agilent, Stockport, UK) equipped with an autosampler (Agilent, Stockport, UK). Each liquid extract (3 µL) was injected in splitless mode onto a DB-FFAP polar column (30 m 0.25 mm I.D., 0.25 µm film thickness, Phenomenex, Macclesfield, UK). The inlet temperature was 240 °C, and the interface temperature was 250 °C. The oven temperature was 45 °C initially, rising by 4 °C/min to 220 °C, and held for 35 min. Helium was used as the carrier gas at 2.2 ml/min. Post column the signal was split equally between the mass spectrometer, the FPD detector (Agilent, UK, operating in sulfur mode) and the odour port (ODP, Gerstel, UK). The mass spectrometer was operated in electron ionization mode with a source temperature of 230 °C, a quadrupole temperature of 150 °C, an ionising voltage of 70 eV, and a scan range from m/z 40 to m/z 300 at 5.3 scans/s. The data were acquired and analysed using Masshunter software (Version 4.5, Agilent, UK). Compounds were identified by first comparing their mass spectra with those contained in the NIST14/Wiley Mass

Spectral Database. Identities were confirmed by comparison of their linear retention index against those of authentic standards.

5.2.6. Calculation of relative recoveries

Relative quantitation was performed using the peak areas of the analytes relative to the peak area of an internal standard (5-methyl-2-hexanone) in the same sample. The relative analyte concentrations were calculated using peak areas relative to the internal standard, and the relative recoveries from SAFE were calculated:

$$\frac{\text{(Relative concentration in the post-SAFE extract)}}{\text{(Relative concentration in the pre-SAFE extract)}} \text{ x } 100$$

These relative recoveries represent how well a single internal standard behaves when a wide range of compounds are analysed in different matrices.

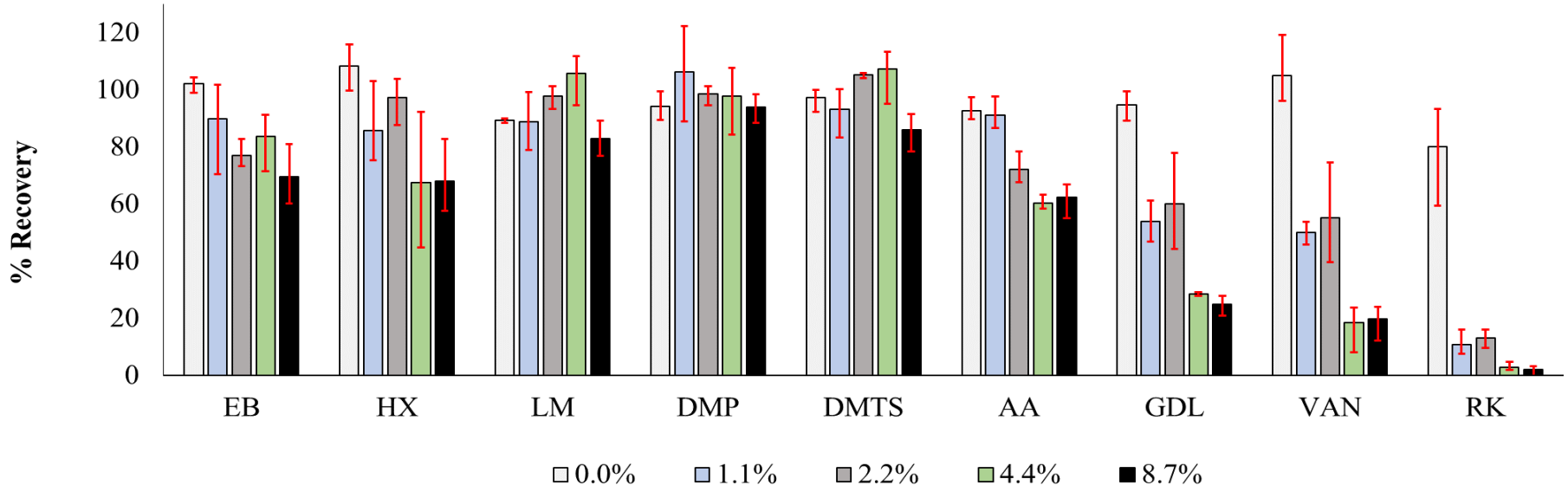
5.2.7. Statistics

The relative recovery data for each compound were analysed by one-way analysis of variance (ANOVA) using XLSTAT statistical and data analysis solution (Addinsoft (2020) New York, USA). For those compounds exhibiting significant difference in the ANOVA, Fisher's least significant difference (LSD) test was applied to determine which sample means differed significantly ($p < 0.05$).

Although cheese is composed of various non-volatile components (including proteins, fats and carbohydrates), the non-volatile material extracted into the organic extract of cheese is likely to be largely composed of fat, as fat is readily soluble in diethyl ether. It is unlikely that other more polar components (proteins, carbohydrates) are present above trace levels, so discussion of these results will focus on fat content as the variable influencing yield of volatile compounds.

5.3. Results

Figure 5.1. Bar graph displaying relative recovery data for volatile compounds in solvent extracts of varying fat content during SAFE.



Compounds are displayed from left to right in order of increasing boiling point, and labelled according to abbreviations listed in table 5.1 Data shown are mean values from data recorded in triplicate, error bars represent the range for each relative recovery data point

The results shown in figure 5.1 (see also table 5.2) demonstrate that fat content affects the relative recovery of volatile compounds during SAFE. For all compounds except limonene and 2,6-dimethylpyrazine there was a significant ($p < 0.05$) decrease in relative recovery when fat content was increased from 0 to 8.8%. As the boiling point increased, the significant difference was observed when fat content was ≥ 2.2% (from anisaldehyde onwards) and a significant ($p < 0.05$) reduction was observed at 1.1% fat for the three highest boiling compounds (γ-decalactone, vanillin and raspberry ketone). The extent of the reduction was also greatest in the high boiling compounds: at 4.4% fat, mean relative recovery for γ-decalactone, vanillin and raspberry ketone were 28, 18 and 3% respectively. Higher boiling point volatiles and higher concentrations of fat in the extract were both associated with substantially lower relative recoveries during SAFE.

5.4. Discussion

5.4.1. Relative recovery from standard in solvent

The results for the 0 % fat sample (standard in diethyl ether) agreed closely with previous work (Engel et al., 1999). In both studies the recoveries from fat-free systems were high, ranging from 80-108 % in the present study and 84–100% in previous work. Neither work suggested that higher boiling point volatiles were less well recovered from the 0 % fat matrix, although this trend was observed when fat was introduced to the matrix. Figure 5.2 displays the yield data reported previously by Engel et al. (1999) compared to the data from this study in relation to the boiling point of the analytes.

3079 Table 5.2. Mean recovery (%) (n=3) and standard deviation of each compound after SAFE from extracts of different fat content

	Fat Content											
Compound	Code	0%		1.1%		2.2%		4.4%		8.8%		Sig[a]
ethyl butanoate	EB	102	(2.9, a)	90	(17.1, ab)	77	(5.05, ab)	84	(10.6, ab)	70	(10.5, b)	*
hexanal	HX	108	(8.1 ,a)	86	(15.0, ab)	97	(8.52, ab)	67	(23.8, b)	68	(13.1, b)	*
limonene	LM	89	(4.9)	89	(16.8)	98	(3.46)	106	(12.0)	83	(5.00)	ns
2,5-dimethylpyrazine	DMP	94	(0.8)	106	(10.0)	99	(3.98)	98	(9.63)	94	(6.17)	ns
dimethyl trisulfide	DMTS	97	(4.2, ab)	93	(8.85, ab)	105	(1.03, ab)	107	(10.5, a)	86	(6.89, b)	*
4-anisaldehyde	AA	93	(4.1, a)	91	(5.83, a)	72	(5.49, b)	60	(2.51, b)	62	(6.37, b)	***
γ-decalactone	GDL	95	(5.1, a)	54	(7.14, b)	60	(16.9, b)	28	(0.69, c)	25	(3.59, c)	***
vanillin	VAN	105	(12.4, a)	50	(3.90, b)	55	(17.7, b)	18	(9.02, c)	20	(6.62, c)	***
raspberry ketone	RK	80	(18.1, a)	11	(4.72, b)	13	(3.21, b)	3	(1.52, b)	2	(1.79, b)	***

3080 Numbers in brackets refer to the standard deviation of the data. The letters refer to significant difference, within each row, values with
3081 the same letter are not significantly different from each other ($P < 0.05$). [a]Probability, obtained from ANOVA, that there is a difference
3082 between means; ns - no significant difference between means ($P < 0.05$); * significant at the 5% level; ** significant at the 1% level;
3083 *** significant at the 0.1% level. At 0% fat content, there was no significant difference in recovery between the compounds, except
3084 for RK which was significantly lower than EB and HX using Fishers least significant difference at p=0.05.

While the majority of the analytes differed between the two studies, vanillin was used in both. Previously, the average yield for vanillin in a 0 % fat matrix was reported as 100 % (Engel et al., 1999), which agrees closely with the average relative recovery of 105 % from this study.

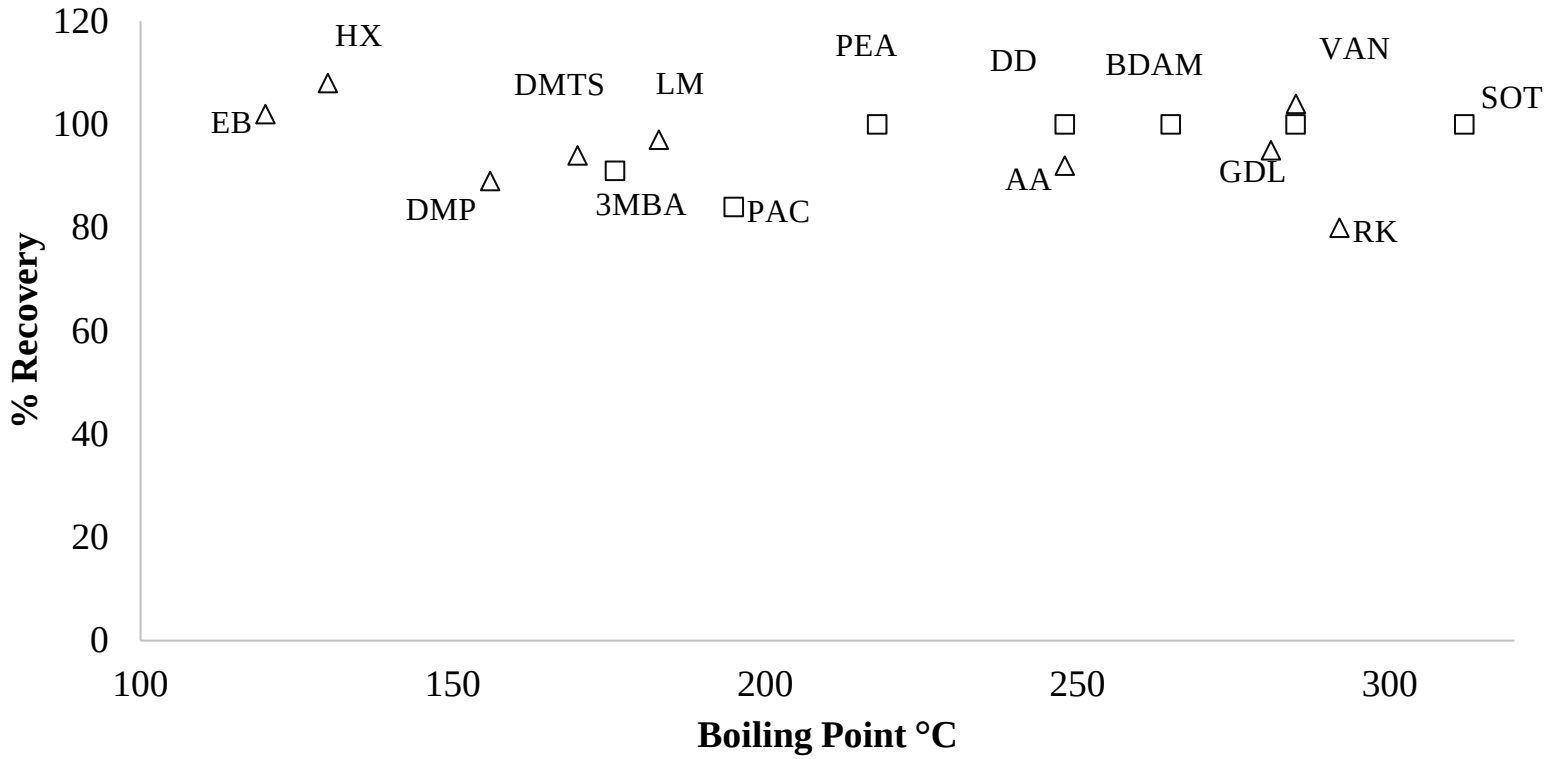
5.4.2. Comparison to previous work on SAFE yields

Engel et al. (1999) showed significantly lower yields were obtained from a high-fat (50 %) extract, especially the higher boiling point compounds. Figure 5.3 shows their yield data from an extract containing 50 % fat extract. The data show a general trend for lower yield at higher boiling points.

The present study extends the results of Engel et al in 50% fat, to demonstrate that even a moderate concentration of fat (up to 8.8%) in a solvent extract can also significantly ($p < 0.05$) affect the yield of volatiles during SAFE. While the previous work used a fat model system comprised of a synthetic mixture of triacylglycerides, the present study used real cheese as the matrix. Further studies would be required to determine whether the fatty acid profile significantly ($p < 0.05$) affects the relative recovery during SAFE.

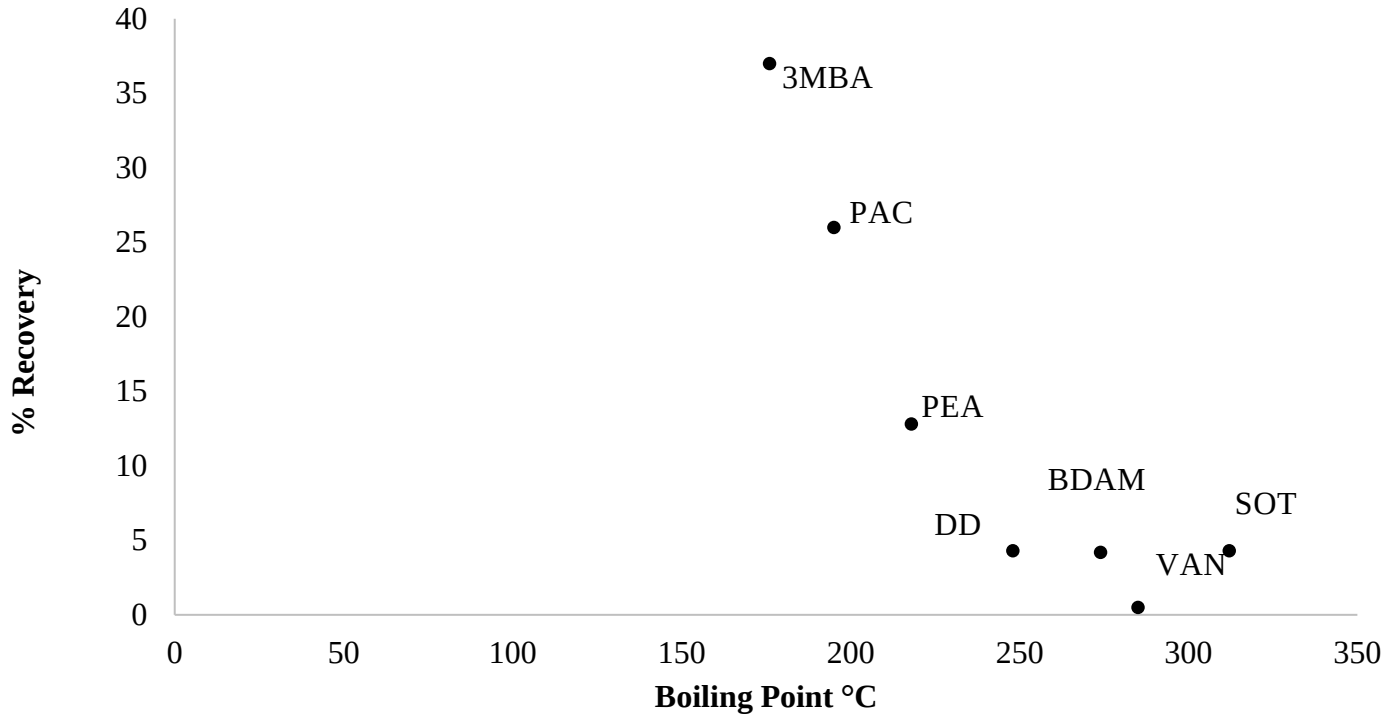
Figure 5.4 highlights the relationship between boiling point and relative recovery of the volatile during SAFE from the 8.7 % fat extract, and shows a similar relationship between relative recovery and boiling point to that observed in the data of Engel et al. There was no evidence that hydrophobicity was related to the relative recovery during SAFE in the present study, for example 2,5-dimethylpyrazine and limonene have Log P values of 0.67 and 4.57 respectively, however, their recoveries from the high-fat cheese extract were very similar.

Figure 5.2. Relative recovery from solvent extracts containing 0 % fat during SAFE.



Data are a comparison of values presented in previous literature □ (Engel et al, 1999) and the present work △ (see table 5.1 for codes). Recoveries are all in the range of 75 to 110% and no visual trends by boiling point are apparent.

Figure 5.3 Relative recovery from solvent extracts containing 50 % fat during SAFE.

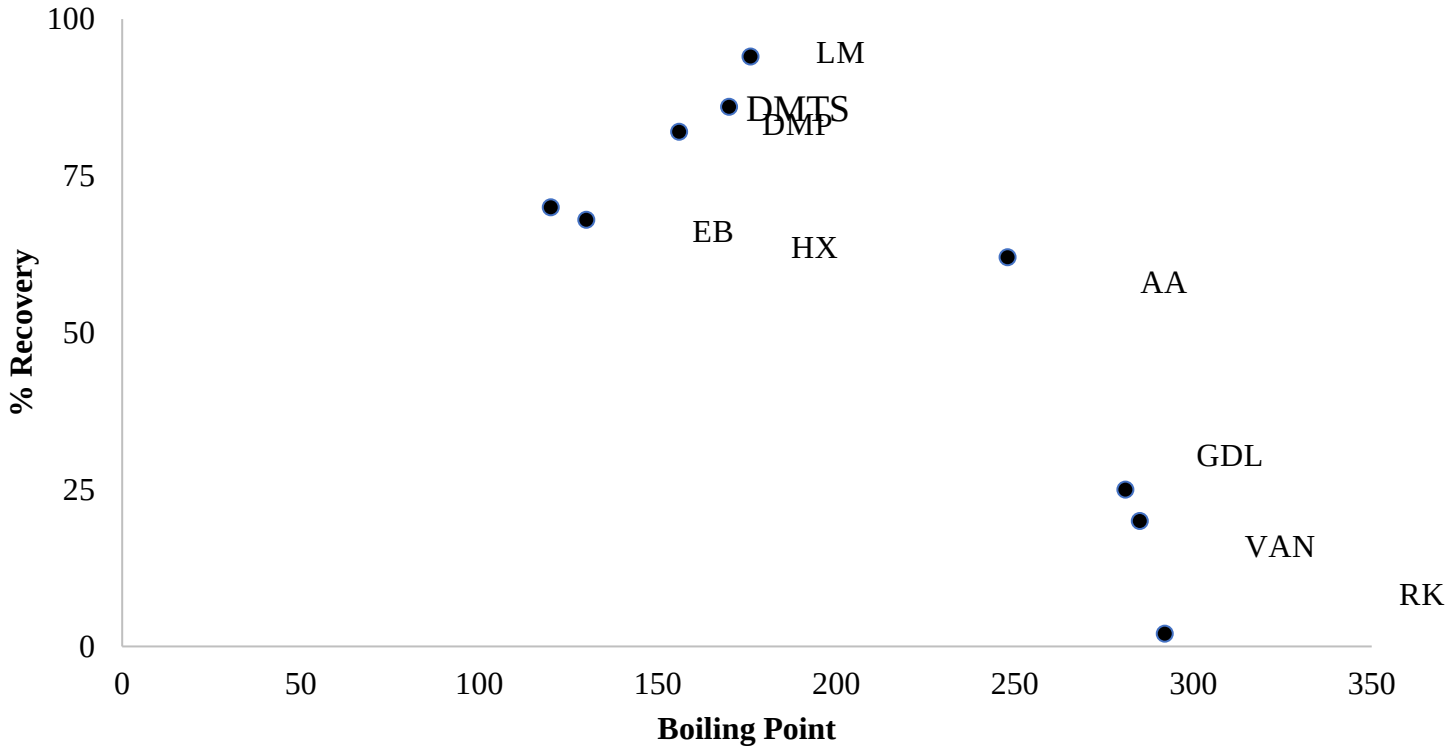


Data taken from a previous study by Engel et al., (1999). See table 5.1 for codes.

The data suggest that at moderate concentrations of fat, boiling point has a much more significant impact on relative recovery during SAFE than hydrophobicity.

An alternative to SAFE, thin layer high vacuum distillation (TLHVD) was reported by Krings, Banavara and Berger (2003) to demonstrate improved recoveries of volatiles from a high-fat (50–90 %) model extract compared to previous relative recovery data for some volatiles using a SAFE method. TLHVD involves the slow movement of a thin film of liquid extract down a warm jacketed condenser into a flask below. The TLHVD system is under high vacuum such that volatiles are evaporated from the thin film and then captured in a series of cold traps. As with SAFE, high boiling point compounds were less well recovered from the TLHVD system compared to low boiling point compounds, and the authors also found Log P to be related to the relative recovery in a high-fat matrix. They reported a good correlation between the product of the boiling point and log P values and the recoveries for the range of volatile compounds, with the exception of lactones. TLHVD may offer a more effective alternative to SAFE for recovery of volatile compounds from fatty matrices. Further work to is required to determine whether dilution of an extract prior to TLHVD can improve the recovery of high boiling point/Log P volatiles, especially lactones. Despite promising data on TLHVD, SAFE is a much more widely used technique and is considered the gold-standard for isolation of volatiles from fatty matrices. As such, SAFE is the focus of this work and the discussion of the literature to follow.

Figure 5.4. Relative recovery from solvent extracts containing 8.7 % fat during SAFE.



See table 5. 1 for codes.

Recently, SAFE recovery data for a number of compounds from a diethyl ether/ dichloromethane (ratio 2:1) bread crumb extract containing a low level of fat (less than 1%) were reported (Pico, Oduber, Gómez, & Bernal, 2018). Given this low level of fat, the recoveries were lower than would be expected when compared to the data from the present study.

For example, the recovery of limonene from the bread crumb extract (less than 1% fat) was 24 %, while we report recoveries of 93-106% from extracts containing 0-8% fat. The authors reported that matrix effects contributed to low recoveries in their study, however, when they adjusted for the matrix effect for limonene the calculated extraction efficiency was still only 63%. Furthermore, the data reported did not show a relationship between boiling point and % recovery.

Though the solvent extract from bread produced by Pico et al was comparable to that reported in this study in terms of fat content, the high starch content of bread may have impacted on the recovery data. Starch has been known to form complexes with volatile compounds (Jeon et al., 2003), especially acids and this may affect solid-liquid extraction.

5.4.3. Significance to quantitation and GC-O studies

In this study the aim was to compare the amount of a range of volatiles in a solvent extract pre- and post-SAFE at various fat contents, rather than to accurately quantify their concentrations. A single internal standard was included to correct for any losses of solvent during SAFE. However, low recoveries of high boiling point volatiles relative to a lower boiling point internal standard demonstrate the inaccuracy of using a single internal standard approach for quantifying a broad range of volatiles in post-SAFE extracts. Better techniques for quantitation in these circumstances are well

known. For example, using multiple internal standards which represent a broader range of volatiles can improve quantitation somewhat, but chemical differences between the chosen standards and the analytes may still introduce inaccuracies. Standard addition of each of the analytes into the sample and multiple levels to develop a standard addition plot is a better approach to quantitation but requires multiple extractions at different concentrations of spiked analytes. Standard isotope dilution assay (SIDA) is the method of choice for quantitation where isotopically labelled standard are available. While dilution of solvent extracts to a low level of fat may significantly ($p < 0.05$) improve the recovery of higher boiling point volatiles, other techniques are recommended if accurate quantitation is required.

The results outlined in this study highlight the challenge of using SAFE for obtaining an extract which is representative of high-fat food matrices such as cheese. When comparing extracts by GC-O using techniques such as AEDA, extracts which contained a moderate or high level of fat during SAFE may contain significantly ($p < 0.05$) lower quantities of high boiling odorants than the original foodstuff, which may prevent their detection during GC-O. For GC-O comparisons to be effective, it is crucial to obtain solvent extracts which are representative of the aroma of the foodstuff. Quantitation of odorants, even using addition techniques such as stable isotope dilution assay (SIDA), and subsequent recombinant studies may fail to entirely recreate the aroma of foodstuffs in cases where key odorants were not detected during GC-O. Other considerations such as choice of extracting solvent, number of aliquots of solvent used during extraction and extraction methods (e.g Soxhlet) may all also influence the recovery of volatiles in the extract. To the authors' knowledge this is the first time a moderate to low fat content in the extract

during SAFE has been reported to impact significantly ($p < 0.05$) on yield of volatiles.

5.4.4. Significance to previously published work on cheese

Considering the results from the present work, it is possible that the significance of compounds with high boiling points in cheese may have previously been underestimated due to a moderate or high concentration of fat in the solvent extract during SAFE/vacuum distillation extraction. Comparison of the relative recovery of vanillin in the present work and the study of Engel et al (1999), recorded at 4.4 % and 50 % fat respectively, indicated approximately a 40-fold difference, which is significant enough to affect detection by GC-O and FD factors calculated during AEDA. The relative recovery of vanillin was also over twice as high in the 1.1 % extract compared to the 8.7% extract, indicating that even a difference of low to moderate fat concentration can influence relative recovery.

Several studies (Carunchia Whetstine, Drake, Nelson & Barbano, 2006; Milo & Reineccius, 1997) have used vacuum distillation techniques to determine the key odorants in cheeses containing high levels of fat. Milo and Reineccius (1997) compared FD factors obtained from full fat and 40 % reduced fat Cheddar. In this study an older version of vacuum distillation was used rather than SAFE, but it has been shown to follow similar trends of low recoveries for high boiling point compounds from fatty matrices (Engel et al., 1999). As such, the vacuum distillation used by Milo and Reineccius (1997) is likely to also have been affected by the fat composition of the solvent extract. Fat contents of the cheeses reported by Milo and Reineccius (1997) were not recorded; however, it is probable that the cheese extracts contained approximately 40 % and 20 % fat respectively due to a difference in the

quantities of the two cheeses used. As the key odorants were also quantified by spiking with deuterated standards, the quantitative differences reported between high and low-fat cheese are robust. However, it is possible that there were odorants present in the high-fat cheese which were not detected due to depletion in the high-fat extract during vacuum distillation.

For example, 6-(Z)-dodecen-γ-lactone reported by Milo and Reineccius (1997) was quantified at a very similar level in both the low and high-fat cheese, while the low-fat FD factor was 4 times that of the high-fat cheese. This is likely to have been a result of depleted recovery during vacuum distillation of 6-(Z)-dodecen-γ-lactone from the high-fat cheese extract, due to the high boiling point of this compound. This low recovery would affect the GC-O FD factor, but not the quantitation due to the robust quantitation method used.

Drake, Miracle and McMahon (2010) also compared FD factors of full and reduced fat cheeses. The extracts of the two cheeses underwent SAFE containing approximately 32 % and 5 % fat respectively, which is likely to have significantly influenced the volatile recovery of the two extraction procedures, especially affecting volatiles with higher boiling points. Furthermore, compounds were quantified by comparison to an external standard curve obtained by spiking standards into water, rather than the cheese matrices, followed by solvent extraction and SAFE.

In light of the results from the present study, recoveries from the high-fat cheese extract are likely to differ significantly from those used to generate the external standard curve and from the low-fat cheese extract. Further investigation into the relative recovery of volatiles from the two cheese matrices may be required to confirm their findings.

A key finding of the work of Drake et al. (2010) was increased burnt sugar notes in the 9-month aged low-fat cheese compared to the 9-month aged high-fat cheese. The authors attributed the burnt note in the low-fat cheese to furanone compounds; furaneol, homofuraneol and sotolone, for which higher FD factors were obtained for the low-fat cheese extract than for the high-fat cheese extract. With the exception of homofuraneol, the differences in FD factors between low and high-fat cheese were very large. For example the FD factor of sotolone was <3 in 9 month aged high fat cheese, but 531441 in the 9 month aged low fat cheese. However, when sotolone was quantified the differences in concentration between low and high-fat cheese were shown to be less than a single order of magnitude.

In light of the results from the present study, the high-fat cheese extract obtained by Drake et al. (2010) is likely to have been significantly depleted of higher boiling point compounds during SAFE. This may have led to artificially low FD factors for furanones in the high-fat cheese, and also have affected the external standard quantitation. As the difference in FD factors between the low and high-fat cheese were so large, the conclusion that furanones contribute to burnt notes in low-fat cheese is likely to be robust. However, depletion of the high boiling point compounds in the high-fat sample may explain the poor correlation between FD factors and concentration.

These authors also discussed lactones as key contributors to milk-fat flavour, however the FD factors for most lactones were similar in the low and high-fat cheeses despite a significantly lower milk-fat score in the sensory study for the low-fat cheese. This sensory difference is a logical result as lactones are derived from triglycerides precursors which might be present in lower amounts in the lower-fat cheese. The results of the present study raise the possibility that the recoveries of

lactones may have been significantly reduced in the extract from the high-fat cheese, leading to artificially low FD factors.

5.4.5. Recommendations

To ensure solvent extracts for GC-O studies are closely representative of foods, we demonstrate that dilution of fatty solvent extracts prior to SAFE significantly ($p < 0.05$) improves yields of high boiling point volatiles. Dilution prior to SAFE would make a sensible addition in studies comparing the key odorants between low and high-fat versions of the same food, such as cheese. Likewise, it has been shown that multiple aliquots of extraction solvent can be used to increase recoveries when extracting volatiles from foods, this approach could also serve a dual purpose of diluting the fat content in the extract prior to SAFE.

Quantitation from fatty matrices during SAFE is best performed by including multiple, appropriately selected, internal standards, ideally C13 labelled analogues of the analytes of interest. However, in the absence of C13 labelled standards, dilution of the extract prior to SAFE may increase recovery of higher boiling point volatiles relative to a single internal standard.

5.5. Conclusion

This study has demonstrated that even low concentrations of fat in the solvent extract can have a significant impact on yields of volatile compounds during SAFE. Higher levels of fat in the solvent extract and higher boiling points of the analytes were both associated with lower relative recoveries during SAFE. Dilution of cheese extracts to a low level of fat led to better relative recoveries of high boiling point volatiles. This approach could enable more accurate comparison of volatile compounds in cheeses of differing fat content. It could also ensure that solvent extracts of high fat

foods, such as cheese, are representative in their aroma for the purposes of GC-O studies. Given the recent focus on the production of fat-reduced alternatives to high-fat foods, these findings are important for comparison of aroma profiles in standard and reduced-fat products.

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Chapter 6 - The effect of fat on cooked cheese aroma

Preface to chapter 6

This study expands on the finding in chapter 3 using the improved SAFE methodology outlined in chapter 5. The goal of this study was to verify the trends relating cooked cheese odorants to fat concentration identified by HS-SPME in chapter 3, and especially to ensure they were not due to matrix effects from the differing fat contents in the cheeses. An initial hypotheses regarding the role of fat in the formation of cooked cheese flavour were tested in this chapter and chapter 3. This chapter also focusses on the identification of compounds formed during the cooking of cheese, including the synthesis of aldol and dioxolane products from the reactions of carbonyl compounds.

Authors' contributions: As the main author on the study, I conducted all material preparation, data collection and data analysis, including GC-O, the syntheses, and wrote the first draft of the manuscript. Amanpreet Kuar assisted me with the SEM study and with the writing of the portions of the manuscript related to SEM. Jane Parker, Colette Fagan and Rosa Sullivan contributed to the study conception and design. Jane Parker provided comments on the draft manuscript and was a GC-O panelist.

This chapter is currently being prepared for submission to Food Chemistry for publication.

Abstract

Cheese is a popular ingredient with a high fat content, that is used in a variety of cooked dishes. This work identifies the odorants in cooked Cheddar and cooked low-fat Cheddar extracted using a solvent extraction followed by solvent assisted flavour evaporation. Many odorants (including Strecker aldehydes, 2-methylketones, esters and aldehydes) were only detected by GC-O in the full fat Cheddar, while phenylacetic acid was only detected by GC-O in the low-fat Cheddar. Furthermore, many compounds differed in concentration between full and low-fat Cheddar. A series of aldol products of Strecker aldehydes were detected in cooked cheese along with dioxolanes from the reaction of dicarbonyls. The role of fat as a precursor for flavour development during cooking in cheeses is discussed, and also possible other contributions of fat to formation of cooked flavour in cheese.

6.1. Introduction

Cheese is an important commodity for the global dairy industry. In 2018, over 30 % of milk produced in the UK was used in cheesemaking (Defra, 2018). Cheese is a popular ingredient in many dishes, where it is often used as a cooked topping, (e.g pizza, pasta bake), as part of sauces (e.g fondue) or cooked alone (e.g raclette, saganaki). The aroma of uncooked cheese has received much attention in the literature and is a balance between the concentrations of different volatile compounds including fatty acids, sulfur compounds, lactones and furanones (Avsar et al, 2004 ; Carunchia Whetstine et al, 2006; Frank et al, 2004; Drake et al, 2010; Kilcawley and O'Sullivan, 2007; Suriyaphan et al, 2001; Zehentbauer and Reineccius, 2002).

When heated, cheese undergoes colour and texture changes which have been attributed to thermally induced reactions such as the Maillard reaction (Wang & Sun,

2003). Furthermore, the high fat content in cheese suggests that lipid-degradation pathways are also likely to contribute to cooked cheese aroma. Despite the potential for flavour formation during these reactions, there is relatively little literature focused on cooked cheese aroma. Dumont et al. (1976) studied the volatile profile of cooked Gruyère, reporting aldehydes, ketones and sulfur compounds among other volatiles. The authors determined that products of protein degradation were important to the aroma of cooked cheese. However, the contribution of fat and its breakdown products to the volatile profile of cooked cheese was less clear.

Bertrand et al. (2011) identified 29 odour active volatiles in cooked processed cheese, of which 13 were present in cooked processed cheese which were not odorants in uncooked processed cheese, including 2-methylpropanal, acetic acid, 3-methylbutanal, 2,3-pentandione, dimethyl trisulfide, 2-acetylpyrazine, phenylacetaldehyde, 2,4-dimethyl-4-hydroxy-3(2*H*)-furanone (furaneol), 3-hydroxy-2-methyl-4*H*-pyran-4-one (maltol) and oxepan-2-one (caprolactone) (although many of these are commonly found in cheddar). However, processed cheese differs from cheese substantially in composition, being typically higher in moisture content and lower in protein. Furthermore, Bertrand et al heated their processed cheese to a maximum temperature of 150 °C for up to 7.5 min, which are much milder heating conditions than cheese typically undergoes during oven cooking. Similarly, previous work on heated mozzarella (Henneberry et al, 2015) was focussed on mild heating conditions and did not include GC-O. Therefore, further work is needed to identify the odorants responsible for cooked cheese aroma.

Chapter 3 outlined a comparison of the volatile compounds found in six cooked cheeses (Parmesan, mozzarella, mature Cheddar, high-fat mild Cheddar, medium-fat mild Cheddar and low-fat mild Cheddar) by headspace solid phase microextraction

(HS-SPME). Chapter 3 describes formation of Strecker aldehydes, pyrazines, unsaturated aldehydes, cyclotene and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone in cooked cheese, and losses of ethyl butanoate and ethyl hexanoate. Furthermore, we reported differences in the concentration of Strecker aldehydes, methanethiol, 2-methylketones and fatty acids between cooked low and high-fat mild Cheddar by SPME.

HS-SPME has previously been used to investigate the profiles of uncooked cheese (Delgado et al., 2010; Frank, Owen & Patterson, 2004; Mondello et al., 2005; Lecanu et al., 2002; Henneberry et al, 2015). However, it is known to suffer from matrix effects when comparing matrices of very different composition (Abilleira et al, 2010; de Grazia et al, 2017). In particular, matrix fat content is known to affect the headspace concentration of volatile compounds according to their hydrophobicity (de Grazia et al, 2017; Sullivan et al, 2021). As the high-fat and low-fat mild Cheddars differ substantially in fat content (~35% and ~2% respectively), a further study was undertaken using a liquid extraction and solvent assisted flavour evaporation (SAFE) methodology. The aims of the study were to compare more quantitatively the volatile compounds detected in low, medium and high-fat cooked mild Cheddar. Furthermore, the aims included using gas chromatography olfactometry to determine whether there were any differences in odorants between the low and high-fat samples.

6.2. Materials and methods

6.2.1. Materials

5-Methyl-2-hexanone, diethyl ether, ethanol, concentrated sulfuric acid, water, sodium hydroxide and sodium sulfate were obtained from Sigma-Aldrich Ltd.

(Gillingham, UK). Isopropyl alcohol, 2,3-butanediol, sodium bicarbonate and all carbonyl compounds used during the syntheses were obtained from Synergy Flavours Ltd (High Wycombe). Liquid nitrogen used during SAFE was obtained from BOC (UK). All reference standards were obtained from Synergy Flavours Ltd (High Wycombe).

6.2.2. Cheeses

As described in chapter 3, three mild Cheddar cheeses of differing fat content, low fat (LF, 2 % fat), medium fat (MF, 22 % fat), high fat (HF, 35 % fat), were produced at the University of Reading's pilot plant facility (Reading, UK).

6.2.3. Cheesemaking

As described in chapter 3.

6.2.4 Cooking of cheese samples

Cheese (~50 g) was cut into 1 cm^3 pieces and blended in an electric blade-based coffee grinder (Sonifer, Amazon, UK) for 15 s to a coarse powder and deposited into a ceramic ramekin (70 mm diameter) which had been lined with a circle of greaseproof paper. The cheese was cooked in the ramekins on the centre shelf of an oven (Neff, UK) at 200 °C for 30 min. This method differed from the method outlined in chapter 3 as this work was carried out first, and the method outlined in chapter 3 was subsequently found to be a more appropriate method method for cooking the non-cheddar cheeses. After cooking the cheese was rapidly transferred into a coffee grinder and ground with liquid nitrogen as described below. The cheese samples lost moisture during the cooking process and 50 g of uncooked cheese generated ~30 g of cooked cheese. The entirety of the cooked cheese sample was ground with liquid nitrogen to a fine powder and extracted in the Soxhlet as described in section 6.2.5.

6.2.5 Cheese extraction

The powdered cheese (50 ± 1 g) was added to the body of a Soxhlet apparatus along with 50 µL internal standard solution (5-methyl-2-hexanone 0.500 % in isopropyl alcohol) and 300 ml diethyl ether. The solvent flask was heated to 40 °C and the Soxhlet was left to circulate for 3 hours (6-7 cycles). As we recently reported (chapter 5), fat concentration in extracts going through SAFE extraction affects the recovery of volatiles, especially higher boiling point volatiles (Sullivan et al, 2021). For this reason, the high-fat and medium-fat Cheddar extract recovered from the Soxhlet was diluted with diethyl ether to an estimated 1% fat (approx. 1600 ml, 1000 ml respectively). The low-fat Cheddar extract was not diluted prior to SAFE as it already contained less than 1% fat by estimation.

6.2.4. SAFE

Samples underwent SAFE extraction using glassware conforming to that described in previous literature (Engel et al, 1999). The method was the same as described by Sullivan et al (2021). The water bath and circulatory water were heated to 40 °C and the cooled flask was submerged in liquid nitrogen. The samples were added dropwise such that consistently low pressure (6–9 x 10^{-4} kPa) was maintained. After SAFE, extracts were concentrated in a Kuderna Danish apparatus at 39 °C to a volume of 2 mL, and dried over sodium sulfate.

6.2.5. GC-MS analysis of volatile compounds

All volatile analyses were performed on an Agilent 7890-5977A GC-MS system equipped with an autosampler (both Agilent, Stockport, UK). Liquid extracts (3 µL) were injected in splitless mode onto a DB-FFAP polar column (30 m 0.25 mm I.D., 0.25 µm film thickness), (Phenomenex, Macclesfield, UK). The oven temperature

was initially 45 °C and increased by 4 °C/min to 220 °C, where it was held for 35 min. Helium was used as the carrier gas at 1.2 ml/min. Post column the flow was split equally between the mass spectrometer, the Flame Photometric Detector (Agilent, UK, operating in sulfur mode) and the odour port (ODP, Gerstel, UK). The mass spectrometer operated in electron ionization mode with a source temperature of 230 °C, an ionising voltage of 70 eV, and a scan range from m/z 40 to m/z 300 at 5.3 scans/s. The scan range was selected to avoid detection of lower molecular weight fragments which may have made chromatographic interpretation more difficult. The data were acquired and analysed using Masshunter software (Version 4.5, Agilent, UK). An alkane standard C5-C25, 10 mg/L in diethyl ether was used as a reference for calculation of the LRIs. Compounds were identified by first comparing their mass spectra with those contained in the NIST14/Wiley Mass Spectral Databases. Identities were confirmed by comparison of their linear retention index against those of authentic standards.

6.2.7. GC-O Analysis

Samples for GC-O analysis were the same as those used for GC-MS. GC Method parameters were the same as described in 6.2.5. A moist make up gas was used to dilute the flow to the ODP. GC-O analysis was conducted in triplicate by three experienced sniffers (more than 20 hours experience performing GC-O each), who described the odors in their own words and recorded the retention times and intensities of each on a 1-4 scale (weak, moderate, strong, very strong). This approach differed from that used in chapter 3 due to differences in the conventions of the two laboratories in which the experiments were primarily conducted. LRIs were calculated with reference to an alkane standard C5-C25 (1 mL), 10 mg/L diluted in diethyl ether. GC-O was repeated by one sniffer on a different column phase (DB-

5MSplus column (30 m 0.25 mm I.D., 0.25 µm film thickness), Phenomenex, Macclesfield, UK) otherwise using the same extraction and chromatographic conditions. Compounds were identified by comparison of their odours with authentic standards or The Good Scents Company Website (TGSC, 2018) and verified by comparison to LRIs of authentic compounds or the NIST Chemistry WebBook library and comparison of the MS to library entries (NIST 2014).

6.2.8 Semi-quantification

The following equation was used for semi-quantitation of each analyte.

Conc. (1) = (single ion peak area (1)* factor (1)) / (single ion peak area (IS) * factor (IS)) * conc. (IS).

where 1 represents an analyte. A single selected ion from the GC-MS chromatogram per analyte was used to perform semi quantitation, relative to that of the internal standard. The 'factor' was calculated from a clean reference spectrum for each analyte, using the equation below. The factor was used to correct the peak area of the single ion chromatogram to the peak area of the full scan chromatogram.

Factor (1) = peak area (1) / single ion peak area (1).

6.2.9 Cryogenic Scanning Electron Microscopy (CryoSEM)

Cryo-SEM was performed to image the microstructure of the cooked Cheddars. The cheeses were mounted onto aluminium cryo stubs which were then secured on a specimen shuttle and plunged into nitrogen slush at -210 ^{o}C. The shuttle was transferred under vacuum to the Quorum PP2000T cryo-SEM preparation chamber (Quorum Technologies Ltd, United Kingdom) and fractured using the knife inside the chamber at -190 ^{o}C. The temperature in the preparation chamber was raised to -90 ^{o}C for 40 min to aid the sublimation of surface ice, and then lowered to -135 ^{o}C

when the samples coated with a thin layer of gold for 80 – 120 s. The shuttle was transferred from the preparation chamber to the SEM chamber which was also held at -135 ^{o}C. The micrographs were captured using the Quanta 600 FEG SEM (FEI, United Kingdom) at an accelerating voltage of 20 kV and various magnification factors (as shown on the images) through the user interface (xT Microscope Server version 2.4).

6.2.10 Syntheses of dioxolanes

Various dioxolanes formed between 2,3-butanediol and carbonyls found in cheese were synthesised to verify their presence or absence in cooked cheese. 2,3-Butanediol (1 mL) and a carbonyl compound (acetaldehyde, propanal, 2-methylpropanal, 3-methylbutanal, phenylacetaldehyde, methional, acetone, 2-heptanone, 2-undecanone) (1 mL) were heated along with 1 drop of concentrated sulfuric acid at 80° C for 30 min. Sodium bicarbonate solution (0.5M, 5 mL) was added to neutralise any remaining acid. The top layer (10 µL) was diluted in diethyl ether (1 mL) and dried over sodium sulfate. The resulting products were characterised by GC-MS to obtain reference MS spectra and LRIs on two columns (DB-FFAP and ZB-5plus).

Analysis were performed on an Agilent 7890-5977A GC-MS system equipped with an autosampler (both Agilent, Stockport, UK). For the ZB-FFAP column analysis, liquid extracts (1 µL) were injected with a 20:1 inlet split onto a ZB-FFAP polar column (30 m 0.25 mm I.D., 0.25 µm film thickness), (Phenomenex, Macclesfield, UK). The oven temperature was initially 45 °C and increased by 4 °C/min to 220 °C, where it was held for 35 min. Helium was used as the carrier gas at 1.2 ml/min. Post column the flow was split equally between the mass spectrometer and the FPD

detector (Agilent, UK, operating in sulfur mode) . The mass spectrometer operated in electron ionization mode with a source temperature of 230 °C, an ionising voltage of 70 eV, and a scan range from m/z 40 to m/z 300 at 5.3 scans/s. For the ZB-5plus column analysis, the conditions were kept the same except that the column was a ZB-5plus column (30 m 0.25 mm I.D., 0.25 µm film thickness) (Phenomenex, Macclesfield, UK), the inlet split was 50:1 and the oven temperature was initially 45 °C and increased by 4 °C/min to 170 °C, then raised by 50 °C/min to 240°C where it was held for 5 min.

Details of compounds synthesised can be found in appendix 10.

6.2.11 Syntheses of aldol reaction products

Various aldol reaction products formed between carbonyl compounds found in cheese were synthesised to verify their presence or absence in cooked cheese. Two carbonyl compounds (all combinations from acetaldehyde, propanal, 2-methylpropanal, 3-methylbutanal, phenylacetaldehyde, methional, acetone, 2-heptanone, 2-undecanone) (200 µL) were mixed in ethanol (2 mL) and potassium hydroxide solution (2M, 2 mL) and vortexed (Fisherbrand, UK) at room temperature for 15 min. The resulting solution was cooled in an ice bath and filtered by gravity (Whatman grade 1 filter paper), washed with ice-cooled ethanol (2 x 2 mL) and then dissolved into diethyl ether (2 mL). The resulting products were characterised by GC-MS to obtain reference MS spectra and LRIs on two columns (ZB-FFAP and ZB-5plus). Analysis was performed as for the dioxolanes. Details of synthesised compounds can be found in appendix 11.

6.3. Results and Discussion

6.3.1 Odorants in cooked high fat Cheddar (HF)

Table 6.1 outlines the odorants detected in the cooked HF sample by GC-O. Overall, 26 odorants were detected in the cooked HF Cheddar. Of those, 9 have been reported as odorants in cooked Cheddar (chapter 3) : 3-methylbutanal, dimethyl trisulfide, butanoic acid, phenylacetaldehyde, 3-methyl butanoic acid, hexanoic acid, 4-Hydroxy-2,5-dimethyl-3-furanone (furaneol), 5-ethyl-4-hydroxy-2-methyl-3(2*H*)-furanone (homofuranol) and 2-methyl-3-furanthiol. 3-Hydroxy-2-methyl-4*H*-pyran-4-one (maltol) has previously been reported in cooked processed cheese (Bertrand et al, 2011). Additionally, 13 compounds have been reported as odorants in cooked cheese for the first time (2-nonanone, 3-octen-2-one, ethyl octanoate, acetic acid, (E)-2-undecenal, 4-methyl-2-phenyl-2-pentenal, 3-hydroxy-2-methyl-4*H*-pyran-4-one (maltol), 5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone (abhexone), (Z)-6-dodecenyl lactone, dodecanoic acid, 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one (sotolone), decanoic acid and 12-methyltridecanal). While many of the odorants are typically found in uncooked cheese (see chapter 2.3.4) (Avsar et al, 2004 ; Carunchia Whetstine et al, 2006; Frank et al, 2004; Drake et al, 2010; Suriyaphan et al, 2001; Zehentbauer and Reineccius, 2002), their concentration was higher in the cooked cheeses than in their uncooked counterparts suggesting that they may be more important to cooked cheese aroma than uncooked cheese.

4-Methyl-2-phenyl-2-pentenal possesses a brown chocolate-like aroma and is an aldol condensation product between 2-methylpropanal and phenylacetaldehyde. Its presence has not been reported previously in cheese, although another aldol product (5-methyl-2-phenyl-2-hexenal) was reported previously in cooked Gruyère (Dumont et al, 1976).

No pyrazines were detected as odorants in this study, despite three pyrazines having been detected in cooked mature Cheddar (chapter 3). In chapter 3, it was noted that pyrazine concentrations were significantly ($p < 0.05$) lower in the cooked mild Cheddar (equivalent to HF in this study) that the cooked mature Cheddar. Differences between the compounds detected may be due to the cheeses used, which was a mature Cheddar in chapter 3 compared to a mild Cheddar in this work. More extensively aged cheeses have higher concentrations of amino acids, which may contribute to higher concentration of amino acid breakdown products (including pyrazines) when cooked.

Furthermore, methional and several other Strecker aldehydes were also not detected in this study having been previously reported as odorants in mature cooked Cheddar (chapter 3). Strecker aldehydes are also formed from amino acids, which may explain their absence in the mild Cheddar due to less advanced maturation processes compared to the mature cheddar analysed in chapter 3. Alternatively, the sampling method (SPME compared to liquid extraction and SAFE) and modification of the oven cooking parameters between the two studied may have resulted in some differences in the compounds detected. Headspace techniques are well suited for the extraction and identification of highly volatile compounds due to the high concentration of volatile compounds in a sample headspace, and the absence of coeluting solvent peaks in the GC-MS chromatogram.

6.3.2 Odorants in cooked low-fat Cheddar (LF)

Table 6.2 shows the list of odorants detected in the LF. Fewer compounds, only nine from twenty-six, were detected in the cooked LF cheese compared to the HF. A comparison of the quantitative data for many of the HF odorants (see section 3.3)

shows that many were significantly ($p < 0.05$)less concentrated in the LF cheese, which is likely to be the reason they were not perceived during GC-O.

However, phenylacetic acid was detected in the LF GC-O and not detected in the HF. Additionally p-cresol was detected at a higher concentration in the LF than HF cooked Cheddar, and is a known source of 'unclean' flavour in Cheddar (Kilcawley, 2017). Both odorants have been reported previously as metabolites of amino acids phenylalanine and tyrosine respectively in cheese (Dunn & Lindsey, 1985; Guthrie, 1993). Their formation in cheese during ripening is driven by microbial catalysis of the amino acids, tyrosine and phenylalanine produced by proteolysis (Gumalla and Broadbent, 2001). Tyrosine is converted into the α-ketoacid (p-hydroxyphenylpyruvic acid) through aminotransferase reactions (catabolized by *Lactobacillus* adjunct cultures), which can be converted through oxidative decarboxylation into p-hydroxyphenyl acetic acid and finally to p-cresol through the action of various cultures (Guthrie, 1993). Phenylalanine can be converted through similar mechanisms to phenylpyruvic acid and phenylacetic acid (Gumalla and Broadbent, 2001).

Phenylacetic acid concentrations were higher in the cooked cheeses than the uncooked cheeses, but similar on a dry-weight basis indicating that the increase is likely to be driven by loss of mass in the cheese during cooking rather than formation of phenylacetic acid. P-cresol on the other hand was much more concentrated in the cooked cheeses than the uncooked cheese, even on a dry weight basis. This suggests that p-cresol formed in cheese by a thermally induced reaction. P-cresol was highest in the LF cooked Cheddar, which would be consistent with formation from the higher concentration of its amino acid precursor in the LF cheese.

3640 Table 6.1. Odorants detected by GC-O in cooked HFC.

				LRI (GC-O)		
No.	Compound	Odor		DB-5	FFAP	
1	3-methylbutanal	chocolate, cocoa	4	653	926	LRI-FFAP, MS,
2	dimethyl trisulfide	sulfurous, unpleasant	4	951	1370	LRI-FFAP, MS,
3	2-nonanone	cheesy	4	1091	1381	LRI-FFAP, MS,
4	3-octen-2-one	mushroom, cheesy	3	1039	1401	LRI-FFAP, odor
5	ethyl octanoate	banana, sweet	2	1194	1431	LRI-FFAP, MS,
6	acetic acid	vinegar, sour	4	625	1436	LRI-FFAP, MS,
7	(E)-2-nonenal	stale, oxidised, cardboard	4	1164	1537	LRI-FFAP, odor
8	butanoic acid	cheesy, sweaty	4	756	1619	LRI-FFAP, MS,
9	phenylacetaldehyde	honey, floral	3	1020	1632	LRI-FFAP, MS,
10	3-methylbutanoic acid	cheesy, sharp	4	834	1662	LRI-FFAP, MS,
11	(E)-2-undecenal	coriander	4		1750	LRI-FFAP, MS,
12	hexanoic acid	Unpleasant, fatty	4	989	1841	LRI-FFAP, MS,
13	4-methyl-2-phenyl-2-pentenal	chocolate, brown	4		1931	LRI-FFAP, MS,
14	3-hydroxy-2-methyl-4*H*-pyran-4-one	cooked, sweet, caramel	4	1127	1977	LRI-FFAP, MS,
15	4-hydroxy-2,5-dimethyl-3-furanone	caramellic	4	1072	2029	LRI-FFAP, MS,

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16	p-cresol	stable, faecal	4		2080	LRI-FFAP, MS,
17	5-ethyl-4-hydroxy-2-methyl-3(2*H*)-furanone	caramel, chocolate	4	1138	2097	LRI-FFAP, odor
18	nonanoic acid	unpleasant, sweet	3	1268	2159	LRI-FFAP, MS,
19	5-ethyl-3-hydroxy-4-methyl-2(5*H*)-furanone	maple, caramel	4		2245	lri, odor
20	undecanoic acid	creamy, waxy	4		2371	LRI-FFAP, MS,
21	(Z)-6-dodecen-y-lactone	creamy, custard	4		2393	LRI-FFAP, odor
22	dodecanoic acid	waxy, creamy	3	1568	2475	LRI-FFAP, MS,
23	3-hydroxy-4,5-dimethylfuran-2(5*H*)-one	maple	4	1116		LRI-5, odor
24	2-methyl-3-furanthiol	meaty	4	902		LRI-5, odor
25	decanoic acid	unpleasant, waxy	4	1375		LRI-5, MS, odor
26	12-methyltridecanal	beef fat	4	1585		lri, odor

Intensity scores were given on a 1-4 scale. [a] Compounds were identified by verifying odour descriptors with The Good Scents Company Website (TGSC, 2018) (Odor), comparison of mass spectra with mass spectra from NIST 11 library (MS) and comparison of LRIs with authentic standards on a DB-FFAP column (LRI-FFAP), a DB-5 column (LRI-5) or the NIST Chemistry WebBook (lri).

Table 6.2. Odorants detected by GC-O in cooked LF.

No	Compound	Odor	Intensity	LRI (GC-O) DB-5	FFAP	Identity based on [a]
1	dimethyl trisulfide	sulfurous	4	951	1373	LRI-FFAP, MS,
2	acetic acid	vinegar,	3	625	1438	LRI-FFAP, MS,
3	butanoic acid	cheesy, sicky	4	756	1623	LRI-FFAP, MS,
4	hexanoic acid	cooked cheese	4	989	1841	LRI-FFAP, MS,
5	4-methyl-2-phenyl-2-pentenal	toasted	3		1950	LRI-FFAP, MS,
6	3-hydroxy-2-methyl-4*H*-pyran-4-one (maltol)	caramel	4	1127	2022	LRI-FFAP, MS,
7	p-cresol	faecal	4		2083	LRI-FFAP, MS,
8	5-ethyl-3-hydroxy-4-methyl-2(5*H*)-furanone	spicy	4		2237	LRI-FFAP, odor
9	phenylacetic acid	honey	4	1020	2568	LRI-FFAP, MS,

Intensity scores were given on a 1-4 scale. [a] Compounds were identified by verifying odour descriptors with The Good Scents Company Website (TGSC, 2018) (Odor), comparison of mass spectra with mass spectra from NIST 11 library (MS) and comparison of LRIs with authentic standards on a DB-FFAP column (LRI-FFAP))

6.3.3 The role of fat content on cooked Cheddar flavour

Quantitative comparisons of a selected series of compounds representing the major compound classes in cooked cheese is shown in figure 6.1. Additionally, quantitative data including statistical comparisons can be found in appendix 9.

Cheese fat is largely comprised of triglycerides of fatty acids. During lipolysis, triglycerides are hydrolysed to free fatty acids (FFA), glycerol, mono and diglycerides through the action of lipolytic enzymes (Fox and McSweeney, 1996). These products of lipolysis, especially the FFAs act as source of uncooked cheese flavour on the own right, and as precursors to formation of other uncooked cheese odorants.

Eight fatty acids (C4-C10, C12) were detected in both the cooked and uncooked cheeses, and lower concentrations were detected in the LF cheese compared to the MF and HF. The even numbered fatty acids were present in higher concentrations than the odd-numbered ones, which is typical of fatty acid compositions of milkfat (Lindmark Månsson, 2008). On a dry weight basis the concentrations in the cooked cheeses were less than 50 % of the concentration in their uncooked counterparts. Although the concentrations of fatty acids in the LF cheese were generally lower than the HF for both uncooked and cooked, the losses of fatty acids during cooking was higher for the HF than LF.

This agrees with previous data on fatty acid concentrations in cooked cheese (chapter 3). Losses of fatty acids during cooking may be due to their participation in reactions or volatile loss. As shorter chain fatty acids did not undergo more substantial losses than those with longer chains, participation in reactions is a more probable explanation than volatile loss.

During cooking, FFAs can as precursors to generation of volatiles, including carbonyl compounds (e.g. 2-methylketones and aldehydes). 2-Methylketones with odd numbered carbon chains from C7-C15 were detected in the cheese. Their concentration was higher in the HF and MF cheese than in the LF, and higher in the cooked cheeses than in the uncooked, which is consistent with their formation from fatty acids during heating from decarboxylation of even-numbered fatty acids (Zabbia et al, 2011). These findings agree with those outlined in chapter 3.

Additionally, a number of unsaturated aldehydes were reported in the cooked cheese, including (E)-2-decenal, (E)-2-dodecenal and (E,E)-2,4-decadienal. These are also products of lipid breakdown during cooking, produced via lipid oxidation. They followed similar trends to the 2-methylketones, their concentration being positively correlated with both higher fat level and with cooking.

Strecker aldehydes were present at much higher concentrations in the cooked cheeses compared to the uncooked, and higher in the HF than LF cooked cheese. The formation of Strecker aldehydes from amino acids and sugars during Strecker degradation is well known, alongside an alternative pathway involving lipid derived precursors (Hildago & Zamora, 2016; Hildago & Zamora, 2019). As the concentration of Strecker aldehydes was highest in the HF cooked cheese, it is possible that fat plays a role in Strecker aldehyde formation in cheese. However, there was a higher sugar concentration in high fat cheese compared to reduced-fat cheese (see chapter 4). Higher sugars, (e.g lactose, glucose or galactose) could also contribute to higher concentrations of Strecker aldehydes.

A series of aldol products of Strecker aldehydes (4-methyl-2-phenyl-2-pentenal, 2-phenyl-2-butenal, 5-methyl-2-phenyl-2-hexenal, 5-methyl-2- isopropyl-2-hexenal,

2-methyl-2-pentenal, 1-ethylidene-3-methylbutanal, 2-methyl-2-butenal) were also detected in the cooked cheese. To the author's knowledge only one of these products has previously been reported in cooked cheese (Dumont et al, 1976). In addition, aldol products of several Strecker aldehydes with 2-methylketones were synthesised, but not detected in cooked cheese with the exception of 3-hexen-2-one. The concentration of aldol products was lower in the LF cheese, which is consistent with the trend observed for the Strecker aldehydes. LRI and MS fragmentation data for the synthesised aldol products are listed in appendix 11.

A series of dioxolanes were detected in the cooked cheeses as reaction products of carbonyls (e.g. Strecker aldehydes or 2-methylketones) with 2,3-butanediol. These products have previously been detected in beer (Peppard and Halsey, 1982) and impart astringent and phenolic flavour notes. The concentration of these compounds were higher in the cooked cheeses, but there was no clear trend for their formation in relation to fat concentration. Peppard and Halsey (1982) found the threshold of 2,4,5-trimethyl-1,3-dioxolanes to be 0.9 mg/L in beer, which is lower than the concentration detected in cooked cheese suggesting that these dioxolanes may contribute to cooked cheese flavour. However, further work is needed to confirm the threshold in a cheese-like matrix. LRI and MS fragmentation data for the synthesised dioxolanes are listed in appendix 10.

Dimethyl disulfide and dimethyl trisulfide were both detected in much higher concentrations in the cooked cheese than in uncooked cheese. Furthermore, their concentration was highest in the LF cheese. In this case the fat in the cheese appears to have an inhibitory effect on the formation of these sulfides. Possible reasons for this could include a higher concentration of amino acids precursors in LF cheese and a role of fat in the structure of the cheese during cooking (see section 3.4). However,

quantitation of volatile sulfur compounds may be less accurate due to their volatility and reactivity.

3-methyl-1,2-cyclopentanedione (cyclotene), 3-hydroxy-2-methyl-4*H*-pyran-4-one (maltol) and 2-acetylpyrrole were all higher in the cooked cheeses than the uncooked, and in the HF compared to the LF. All three of these compounds are sugar degradation products, and their higher concentration in the HF cheese is likely to be due to a higher concentration of sugar in the uncooked HF (see chapter 4).

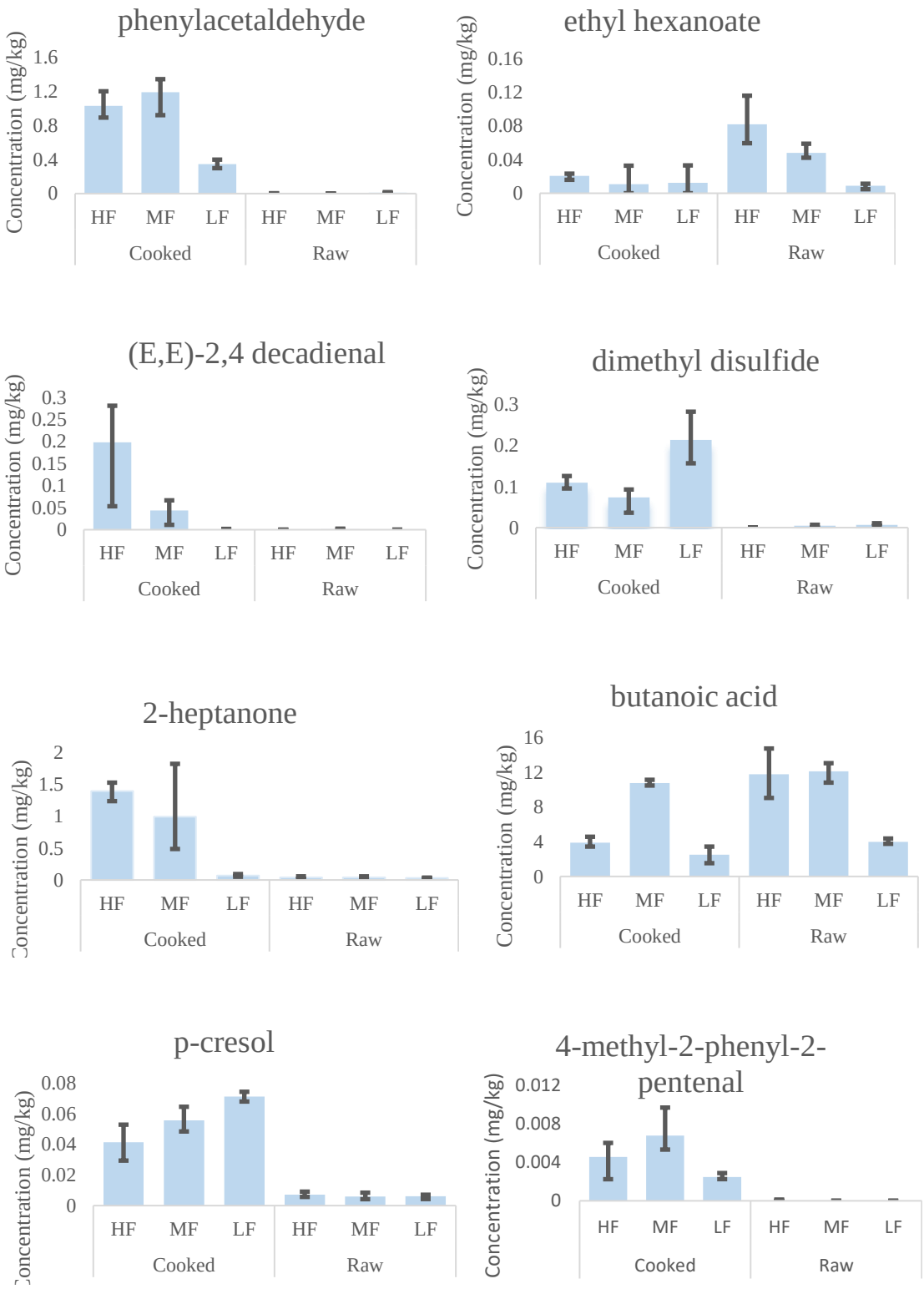
Several esters were detected in the cheeses. Ethyl hexanoate decreased during cooking, but ethyl butanoate concentration remained constant and ethyl acetate increased in concentration. Previous work has found that esters decrease in concentration during cooking, driven by volatile loss (chapter 3). The increase in concentration of ethyl acetate is unexpected as ethyl acetate is a very volatile ester. Formation of ethyl acetate could occur between ethanol and acetic acid which is present in the cheese, however, other acids such as butanoic and hexanoic are also present so formation of other ethyl esters would also be expected.

Acetoin concentration also decreased during cooking. This may be due to volatile loss or involvement of acetoin in thermally induced reactions.

6.3.4 Cheese SEM

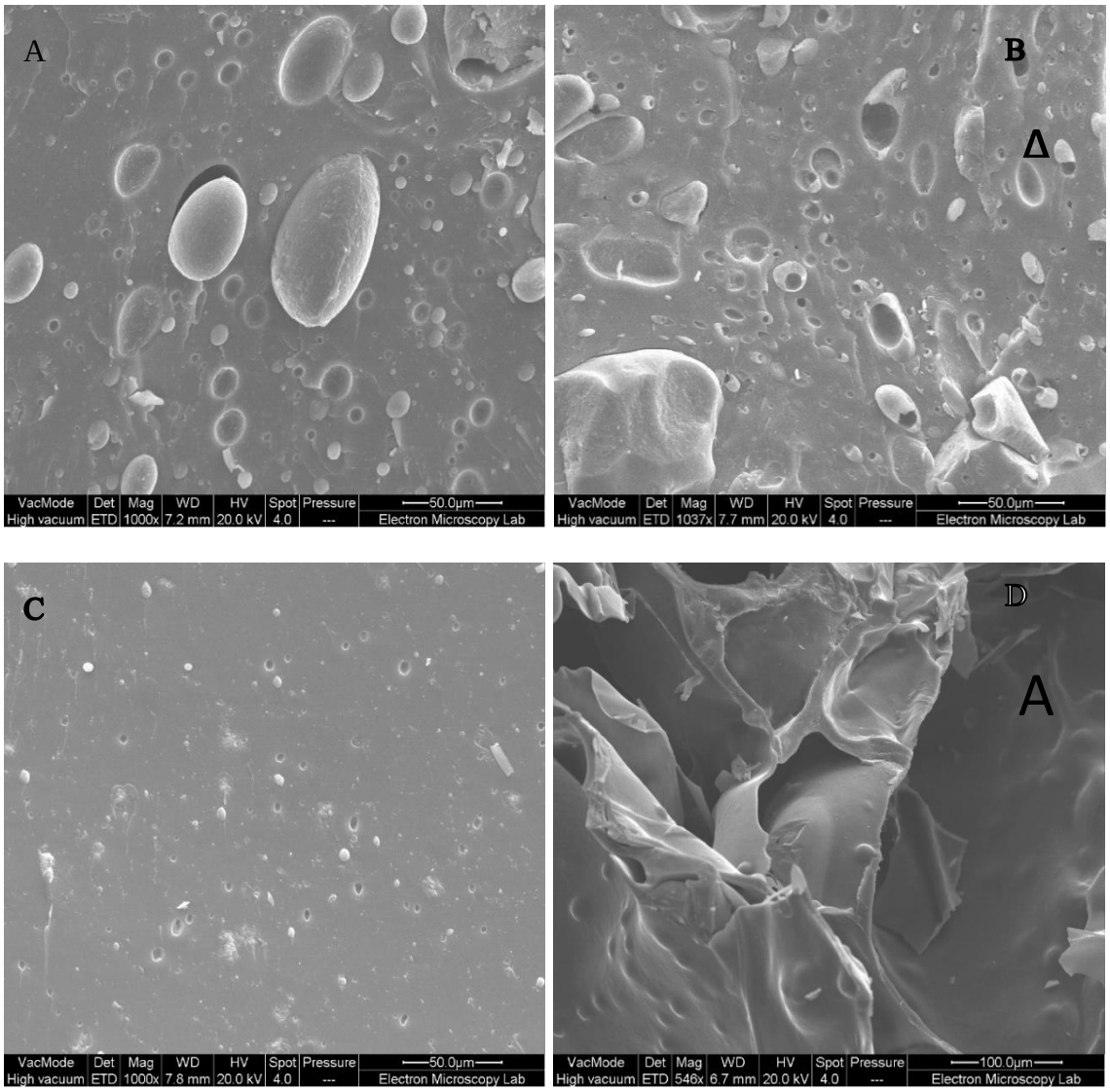
The structure of cheese may be described as a three dimensional casein gel (comprising proteins, water, and dissolved solids) disrupted by globules of fat (Guinee et al., 2000). In reduced fat cheeses, the casein network is typically stronger due to fewer and smaller fat globules dispersed within it, leading to firmer and more rubbery texture in uncooked low-fat cheeses (Mistry, 2001).

Figure 6.1. Bar graphs of semi-quantitative results in uncooked and cooked cheeses.



Error bars in figure 6.1 represent the range of the triplicates.

Figure 6.2. Scanning electron microscope (SEM) images of cooked cheeses.



Letters in figure 6.2 refer to A – HF cooked Cheddar, B – MF cooked Cheddar, C – LF cooked Cheddar (bulk cooked cheese), D – LF cooked Cheddar top.

Figure 6.2 shows examples of SEM images of HF (A), MF (B) and LF (C and D) cooked cheese. During cooking, the HF and MF cheeses appeared visibly very similar in terms of melt, free-oil pooling and colour change. In contrast the LF cheese had visibly little free-fat, a raised skin several centimeters above its surface, and browned

much more extensively than the HF and MF cheeses. This is in line with performance of other low-fat cheeses during baking reported in the literature. Rudan and Barbano (1998) reported similar raised skin formation in a low-fat mozzarella during baking. They attributed it to increased moisture loss at the cheese surface, due to an absence of free-oil.

The HF and MF cheeses had fat globules which were larger and less uniform in shape than the LF cheese, which is consistent with previously studies on uncooked cheese microstructure (Rudan and Barbano, 1998; Guinee et al, 2000). However, the LF structures were visually dissimilar to the MF and HF. The majority of the LF cheese resembled image C, which shows a relatively un-disrupted casein network with small and spherical fat globules. Image D shows the structure of the skin on top of the LF cooked Cheddar. It was very different to the bulk LF cooked cheese and to the other cooked cheeses. There appears to be no fat globules interrupting the casein structure. As suggested in other studies, the lack of free-oil at the surface of the cheese is likely to have promoted rapid browning and thermally induced reactions.

Differences in the structure of the cheese may impact flavour formation during cooking. Dimethyl trisulfide was highest in concentration in the LF cooked Cheddar. While protein concentration in LF cheese is higher than HF and MF cheeses, the increase in dimethyl trisulfide concentration is higher than the increased protein concentration. Dimethyl trisulfide is formed from methionine breakdown (Zabbia et al, 2011). Additionally, the decrease in free-oil at the cheese surface and the rapid loss of surface moisture forming a brown skin may indicate more rapid progress of the Maillard reaction which may lead to increased formation of products such as dimethyl trisulfide (Guinee, 2002).

6.4. Conclusion

Fat contributes substantially to the formation of cooked cheese flavour during cooking. While 26 odorants were detected in cooked HF, only 9 were detected in LF. Notably, 2-nonanone, 2-(E)-2-nonenal, (E)-2-undecenal, nonanoic acid, decanoic acid, undecanoic acid and dodecanoic acid were all products of lipid degradation which were found as odorants in cooked HF but not LF. Furthermore, the concentrations of 2-methylketones, aldehydes and fatty acids were substantially lower in the LF cooked cheese than MF or HF, in many cases significantly ($p < 0.05$) so. We can confirm that lipids in cheese are an important source of precursors to the formation of flavour during cooking.

Lipid precursors have been shown previously to participate in Maillard reaction such as Strecker degradation. Products of Strecker degradation (Strecker aldehydes and related aldol condensation products) were higher in the HF cooked cheese, which is consistent with the presence of lipid-maillard interactions, but may also be attributed to the effect of higher sugar concentration in the HF cheese. The significance of lipid-Maillard interactions for cooked cheese flavour is a topic for further study.

Dimethyl trisulfide was significantly ($p < 0.05$) higher in the LF cheese than the MF or HF cheese. Dimethyl trisulfide is formed from the breakdown of methionine. Although the LF cheese contained higher levels of amino acids, this difference was not enough to explain the higher level of dimethyl trisulfide when cooked. Fat is known to affect the structural changes which occur during cooking in cheese. Specifically, the fat is believed to interrupt the protein phase which may slow Maillard reactions, and to form a layer of free-fat on top of the cheese which protects it from rapid dehydration and browning reactions during cooking (Mistry, 2001). We

believe that the low fat content in LF cheese and absence of fat interruption to the protein phase and free fat layer may contribute to more rapid Maillard reactions when cooked, leading to formation of higher levels of dimethyl trisulfide.

These data may have implications for the cheesemaking industry, in assisting with the development of low-fat cheeses for cooked applications. The scarcity of fatty acids precursors may be compensated to some extent by the use of starter cultures which speed up lipolysis during aging. Additionally, cooking at lower temperatures and various fat replacers have been suggested as tools to compensate for the structural differences between high and low fat cheeses during cooking (Mistry, 2001). With further study, these strategies may also help produce low-fat cheeses with better taste properties when cooked.

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Chapter 7 – Concluding remarks

This research has characterised volatile and non-volatile flavour changes that occur when cheese is cooked. It compares the volatile and selected non-volatile compositions of traditional mozzarella, Parmesan and mature and mild Cheddar, cooked and uncooked. In addition, the role of fat in flavour development during cooking was investigated in a mild Cheddar. Finally, in the course of producing this work the limitations of using SAFE for the comparison of low-and-high-fat matrices were explored and a proposal made for a dilution approach for high-fat extracts prior to SAFE. A summary of these three aspects of the thesis is given below, along with some discussion of limitations and suggested directions for future work for each.

7.1 The flavour of cooked cheese

Substantial differences were found between the presence and concentration of volatile compounds in cooked cheese compared to uncooked cheese. Pyrazines, unsaturated aldehydes, aldol reaction products, furanones, pyranones and cyclotene were detected in cooked cheese but not in uncooked cheese. Furthermore, during GC-O many compounds (including 3-methyl-2-butene-1-thiol, 2-heptanone, furan-2-yl-methanethiol, cyclotene , 3-ethyl-2,5-dimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, 2-methyl-3-methyldithiofuran, (E)-2-decenal, 12-methyl-tridecanal and 4-methyl-2-phenyl-2-pentenal) were perceived as odorants that have not previously been reported in uncooked cheese.

These findings support the hypothesis that cooking cheese leads to formation of odorants not present in uncooked cheese. However, in many cases the difference between uncooked and cooked cheese was in the concentration rather than the presence or absence of odorants. Many compounds (including Strecker aldehydes,

thiols, ketones, furanones and p-cresol) have previously been reported as odorants in uncooked cheese, but were significantly ($p < 0.05$) more concentrated in cooked cheeses than uncooked during this study. Additionally, many of the esters and fatty acids which are characteristic of uncooked cheese flavour were significantly ($p < 0.05$) lower in concentration in cooked cheese than uncooked.

There is evidence to support the hypothesis that lipid and Maillard pathways occur in the formation of odorants in cooked cheese. Both amino acids and sugars decreased in concentration during cooking, which coincided with formation of known Maillard reaction products such as many listed above. Furthermore, the decrease in amino acid concentration during cooking was sufficient to leave some amino acids below threshold in cooked cheeses, which had been above threshold in uncooked cheese.

Similarly, γ-glutamyl dipeptides responsible for kokumi taste in uncooked cheese decreased significantly ($p < 0.05$) in concentration during cooking, supporting an initial hypothesis of this study. Although this difference was not sufficient to change whether any of the peptides were above their taste threshold, this is because the cheese which was cooked was a mild cheese with relatively low levels of γ-glutamyl dipeptides when uncooked. During aging of the mild Cheddars, γ-glutamyl dipeptides increased in concentration substantially, so it is likely that γ-glutamyl dipeptides are important to the flavour of some cooked aged cheeses.

While amino acid and short-chain peptide concentrations increased during aging, sugar concentrations decreased. Pyrazines were detected in cooked mature Cheddar, but not in cooked mild Cheddar or other cooked cheeses. As both sugars and amino acids are precursors to pyrazine formation, it may be that less extensively aged

cheeses (e.g mild Cheddar, mozzarella) have too low an amino acid concentration to generate detectable levels of pyrazines, while very extensively aged cheeses (e.g Parmesan) don't generate high concentrations of pyrazines because they have low concentrations of sugars.

Diketopiperazines were detected in suprathreshold concentrations in some cooked cheeses, having been found at far-below-threshold concentration in uncooked cheese previously (Roudot-Algaron, 1993). Diketopiperazines were more highly concentrated in cooked aged cheese than cooked young cheese, which was attributed to higher presence of short-chain-peptide precursors in extensively proteolyzed cheeses.

Overall, the age of the cheese a highly important factor in determining the flavour when cooked. The carry-over of flavour developed during aging into cooked aged cheeses is unsurprising, but the higher concentration of precursors to cooked flavour development in aged cheeses is worthy of further study.

GC-O was performed on both a mature Cheddar (headspace SPME-GC-O) and a mild Cheddar (SAFE extraction followed by GC-O). Similar odorants were found in the two studies despite differing extraction techniques, although SPME was more effective at detecting very early eluting compounds as their peaks did not coelute with any solvent peaks. Three pyrazines (trimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine) were detected by SPME-GC-O in the mature Cheddar, but as these compounds were not found in the mild Cheddar by SPME it is clear that this difference is due to a difference in the cheese rather than the methodology.

Overall, SPME and SAFE methodologies complemented each other in this study, although the GC-O approached taken were slightly different which precludes an entirely direct comparison. SPME was more effective for the detection of highly volatile compounds, while the SAFE methodology detected aldol reaction products. Additionally, the SAFE methodology facilitated long-term storage of the cooked cheese extract allowing repeated analysis and GC-O studies.

7.1.1 Contribution to knowledge

This work has identified the volatiles responsible for cooked Cheddar aroma. This knowledge is valuable for the dairy industry, as these volatiles can be used as markers for cooked Cheddar flavour in future studies. Furthermore, it has indicated some non-volatile components of cheese which are important precursors to flavour formation during cooking. This knowledge could be used to guide development of new cheeses for cooked applications, for example in the selection of culture and maturation parameters.

The concentration of amino acids, sugars and short chain peptides are all likely to contribute significantly ($p < 0.05$) to flavour formation during cooking. The concentration of these precursors should be managed to achieve a desired effect when the cheese is cooked. In particular, DKPs are present at significantly ($p < 0.05$) higher concentrations in cooked aged cheeses than cooked young cheeses. As DKPs are bitter and metallic in taste, younger cheeses may be chosen for cooking to avoid development of excessive bitter flavour.

Finally, these results may be of interest and of use to the flavour industry. Cheese flavourings are often used to enhance the flavour of cheese sauces, snack seasonings and in dairy-free cheese substitutes. The cheese flavours used typically have an

uncooked profile, but for cooked applications authentic cooked-cheese profiles are desirable. This work outlines the odorants responsible for cooked cheese flavour and may be used by the flavour industry to guide development of new cooked cheese flavourings.

7.1.2 Limitations

Only a small selection of cheeses were included in this study. These were the most popular cheeses in the UK and often included in cooked foods (mild Cheddar, mature Cheddar, mozzarella, Parmesan). While this selection was made to include cheeses of varying maturity and type, it is a limitation of this study that other cheeses that are often cooked (such as Gruyère or halloumi) could not be studied.

GC-O was only performed on Cheddar cheeses (mild high-fat, mild low-fat, mature high-fat). Given the knowledge acquired through this study that the aging period of the cheese is very important to the development of precursors which can be converted to odorants during cooking, it's a limitation of this study that neither a very young (e.g mozzarella) nor very mature (e.g Parmesan) cheese underwent GC-O.

The high-fat, medium-fat and low-fat Cheddars produced during this project were aged for 24 months with sampling at regular intervals to follow the formation of amino acids and γ-glutamyl dipeptides during aging. In hindsight, it would have been interesting to be able to cook the cheeses aged for different periods and analyse for their key volatile and non-volatile constituents. Due to limitations in the quantity of cheese aged for each time period, this analysis could not be performed.

For the non-volatile analysis, analytes were chosen for study based on their predicted importance to flavour change in cheese during cooking. The reactions occurring in peptides during cooking are likely to be very complex and due to the very large

amount of time which would be required to analyse all peptides in cheese, this study only investigated the concentrations of amino acids, selected γ-glutamyl dipeptides and selected diketopiperazines during cooking.

The cooking method chosen, oven cooking, was selected to replicate the most common cooking method used for cheese containing ready-meals and pizzas. Nevertheless, a limitation of this study is that other cooking methods were not explored. For example, using an enclosed system to cook the cheese would minimise volatile losses during cooking.

7.1.3 Directions for future study

The following topics would make interesting extensions to this work:

- The analysis of other cooked cheeses such as Gruyère or halloumi, and GC-O analysis on cooked cheeses other than Cheddar.
- Increase the number of individual cheeses of each type analysed to ensure the results are representative.
- The comparison of different cooking techniques or temperatures.
- Further exploration of the effect of cooking on peptides, including longer chain peptides and their possible contribution to taste.
- The volatile analysis of cheeses from the same batch aged for different maturation periods. This project suggested that some volatiles are formed more extensively in aged cheeses but this is based on comparison of different cheeses with different manufacturing conditions.
- Sensory studies on cooked cheese would confirm the relationship between the analytical findings explored in this thesis and the flavour of cooked cheese. In particular, sensory on cooked cheese could confirm the importance of

bitterness to cooked cheese flavour, which may relate to the formation of DKPs during cooking.

- Sensomics would be an interesting approach to take for confirming the importance of volatiles in cooked cheese flavour. However, a model system which closely represents the cheese matrix would need to be produced to support the sensomics approach.

7.2 The role of fat in cooked cheese flavour

Overall, the role of fat in cooked cheese flavour can be summarised into four potential contributions, which are outlined below.

7.2.1.1 Fat affects aging pathways leading to development of important precursors

Firstly, fat affects the progress of various chemical pathways during the aging process in cheese, which generate precursors that go on to produce flavour compounds during cooking.

Although all of these factors are dependent on processing conditions, generally speaking products of lipolysis, such as fatty acids, are substantially lower in low fat cheeses. Products of proteolysis, short-chain peptides and amino acids, are higher in low-fat cheeses due to the higher protein content and faster rate of proteolysis in low-fat cheeses. Reducing sugars such as a lactose are present at lower concentrations in low-fat cheeses, as lactose breakdown is more rapid in low-fat cheeses.

This contribution of fat to aging is complex, as reducing fat decreases the concentration of some precursors to thermal-induced reactions (free fatty acids, sugars) and increases the concentration of others (free amino acids, short chain

peptides). During cooking, these undergo thermally induced reactions which produce flavour compounds.

For example, the higher lactose concentration in the high-fat uncooked cheese is likely to have contributed to the higher concentration of many sugar-derived volatiles in cooked high-fat cheese (including 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, 3-methyl -1,2-cyclopentanedione, 3-hydroxy-2-methyl-4*H*-pyran-4-one and 2-acetylpyrrole). In the low-fat cooked cheese these sugar-derived compounds were lower in concentration, which is likely to affect perceived flavour as 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, 3- methyl -1,2-cyclopentanedione and 2-acetylpyrrole were identified as odorants in high-fat, but not in low-fat cheese.

7.2.1.2 Fat is a source of precursors to lipid degradation

The fatty acids which are products of lipolysis had lower concentrations in the low-fat uncooked cheese, a trend which was maintained in the cooked cheese. Furthermore, as fatty acids are precursors to lipid degradation reactions which occur during cooking, the products of fatty acid degradation (2-methylketones, aldehydes, lactones) were also lower in low-fat cooked cheeses. 2-Nonanone, (E)-2-nonenal, (E)-2-undecenal, nonanoic acid, undecanoic acid, decanoic acid, dodecanoic acid and 12-methyltridecanal were all lipid-derived odorants in high-fat cooked cheese, but not detected as odorants in the low-fat cooked cheese.

7.2.1.3 Fat may act as a source of precursors to lipid-Maillard interactions

Additional to the formation of 2-methylketones and aldehydes, lipid degradation products may also interact with Maillard degradation pathways to form lipid-Maillard degradation products. The high concentration of Strecker aldehydes and aldol reaction products in high-fat cooked Cheddar would be consistent with this

theory, however, the higher concentration of sugars in the high-fat cheese is also likely to have contributed to Strecker aldehyde formation. More work is needed to confirm the extent of lipid-Maillard interactions to cooked cheese flavour.

7.2.1.4 Fat has a structural role in cheese during cooking, which may influence flavour formation.

Finally, fat is known to play a role in the structure of cheese during cooking. The scanning electron microscopy performed on each of the cooked cheeses demonstrated that in the low-fat cheese there were very few and small fat globules interrupting the casein phase. Furthermore, the surface of the low-fat cooked cheese was structurally highly dissimilar to that of other cheese samples.

The rapid dehydration and browning reported at the surface of low-fat cheeses during cooking has been attributed to Maillard reactions. Some reaction products, such as sulfur compounds (e.g dimethyl trisulfide) were found to be higher in the low-fat cooked cheese than the high-fat cooked cheese. This higher concentration of sulfur compounds may be due to the absence of fat globules affecting the cheese structure or free-fat at the cheese surface.

Comparison of cheese with differing fat concentrations was initially undertaken by SPME, however, it was unclear whether some trends observed may have been caused by matrix effects during the SPME extraction. The samples were extracted again using a SAFE methodology developed for the comparison of low-and-high-fat matrices. The SAFE extraction largely produced similar trends to previous SPME work.

7.2.2 Contribution to knowledge

This study has identified some compounds which may be responsible for flavour differences in using low-and-reduced-fat cheeses for cooked applications. These data may be used by the dairy industry to guide development of new low-fat cheeses to be sold into the food-service sector, for example for use in pizzas and ready-meal sauces.

One of the key findings of this study has been the effect of reduced sugar concentration in low-fat cheeses on development of odorants during cooking. Dairy manufacturers may find that altering cultures to minimise lactose degradation, or opting for shorter maturation periods when using low-fat cheeses for cooked applications will improve generation of aroma compounds during cooking.

Lipid-derived flavour compounds are key to cooked cheese flavour and are present in lower concentrations in low-fat cheeses. Dairy manufacturers may use these data to select cultures with greater lipolytic ability in order to improve low-fat cooked cheese flavour.

7.2.3 Limitations

Although efforts were made to reduce the effect of fat content in maturation related changes in the cheeses by limiting maturation time to 3 months, there were still changes which occurred during maturation related to the fat concentration of the cheeses. These changes, while interesting and relevant for the dairy industry, limited the extent to which the role of fat itself during cooking could be studied. For example, the formation of Strecker aldehydes was higher in the high-fat cheeses than the low-fat, which could be attributed to the role of fat as a Strecker aldehyde precursor (through formation of lipid-derived carbonyls). However, due to the role of fat in

inhibiting lactose degradation during maturation, the high-fat cheese contained a significantly ($p < 0.05$) higher concentration of lactose than the low-fat cheese. As lactose is also a potential precursor to formation of Strecker aldehydes, it is not possible from the current data to be certain whether lipid sources are a significant ($p < 0.05$) contributor to the formation of Strecker aldehydes during cooking in cheese.

As with the previous section, this work focussed only on Cheddar cheese, although other reduced-fat cooking cheeses (especially mozzarella) would also be interesting and relevant topics for the dairy industry. The literature on the subject of reduced-fat mozzarella for pizza toppings has documented the effects of reduced-fat mozzarella on free-fat formation and on structural and browning properties of the cheese (Mistry, 2001). One published solution to this issue is to spray the surface of reduced fat mozzarella with an oil to create a simulated free-fat layer (Rudan and Barbano, 1998). It would be interesting to investigate the possible effects of this approach on flavour formation during cooking on mozzarella.

7.2.4 Directions for future research

Two approaches could be taken to investigate the role of fat in cooking as opposed to cooking and maturation combined.

A model system to replicate cheese could be developed, which could then be studied with varying concentrations of fat and standardised concentrations of other components such as sugars and amino acids. However, it would be important but challenging to ensure that the model system closely replicated the cheese matrix. Alternatively, it may be possible to develop a method to defat high-fat cheese prior to cooking.

Investigations into the role of fat during cooking for other cheese types, especially mozzarella, would be interesting and relevant to the food industry. In particular, the efficacy of spraying the surface of reduced fat mozzarella with oil during cooking on flavour formation would be a valuable subject for research.

7.3 SAFE as an approach for comparing matrices of differing fat concentrations

Although not an initial aim of this study, in the process of evaluating SAFE as a methodology for comparing low-and-high-fat cheese extracts a modified SAFE approach was developed, resulting in a publication.

When performing analysis on complex matrices such as food products, it is important to consider whether the matrix may affect the efficacy of the extraction procedure. This is especially important when comparing products with very different matrices, such as low-and-high-fat versions of a food. Both SPME and SAFE were used during this work, and both are susceptible to matrix effects. In particular, this work highlighted that even a relatively low level of fat in a solvent extract (<10%) can significantly reduce yields of volatile compounds during SAFE.

A solution to this challenge was to dilute the extracts prior to SAFE to a very low (<1%) concentration of fat. This approach was used in the comparison of low-fat, medium-fat and high-fat cooked Cheddar and GC-O of high-fat and low-fat samples. By comparison of previously obtained SPME cheese data with additional data from the modified SAFE method, many of the trends could be confirmed as genuine effects of fat concentration on cooking cheese. Obtaining data from multiple different extraction techniques is good practice to ensure robust conclusions.

7.3.1 Contribution to knowledge

These insights are likely to be relevant for future dairy studies into volatiles in cheeses of differing fat content. Furthermore, the scope of these findings are relevant for any researchers using the SAFE methodology to compare full-and-reduced-fat matrices. To do so robustly, the extracts should be diluted prior to SAFE to comparable and very low concentrations of fat.

7.3.2 Limitations

This work covered only a small set of compounds with varying volatilities and hydrophobicities, as a representative sample. That being said, there may be compounds with volatilities and hydrophobicities outside the range studied, which may behave differently during SAFE depending on fat content.

Although this work is likely to have relevance for other food matrices which contain fat, it would be interesting to explore whether the ratio of saturated to unsaturated fats affects the poor yields of high-boiling point compounds from SAFE of high-fat matrices. As the fat in cheese is relatively saturated, this work is limited to the study of relatively saturated fat on the efficacy of SAFE.

A more robust approach to the quantitation of compounds from matrices of differing composition would be to spike them with isotope labelled versions of each analyte as internal standards. This approach, though robust, was too time-consuming and costly for the limitations of the study, due to the need to obtain or synthesise labelled standards for all analytes. It is hoped that the approach outlined in this study will be beneficial for other researchers and especially those working in industrial research laboratories where obtaining labelled standards is often not feasible. Furthermore,

this approach is more suitable for obtaining a representative solvent extract for the purposes of GC-O than using labelled standards.

7.3.3 Directions for future research

This work could be extended by exploring the dilution approach for a broader range of volatiles and matrices.

7.4 Conclusion

This thesis has documented the characterisation of the flavour of cooked Cheddar, mozzarella and Parmesan. We have shown that cooking cheese changes the concentration and, in some cases, the presence of odorants and volatile compounds compared to uncooked cheese. Furthermore, we have identified changes in the concentration of selected non-volatiles during cooking.

This work has shown the role of fat in cooked cheese flavour to be complex. Lipid and Maillard-derived compounds both contribute to cooked cheese aroma, while lipid-Maillard interaction products may also contribute. The fat level in cooked cheese affects the concentration of non-volatiles, including amino acids and sugars. Fat contributes to the formation of cooked cheese flavour as a precursor to lipid-derived volatiles, by influencing the formation of various tastants and flavour precursors during cheese aging and by its impact to the structural changes occurring during the cooking of cheese.

The dairy industry may improve performance of low-fat cheese for cooked applications by selection of cultures and cheesemaking conditions to slow the progress of lactose metabolism and proteolysis, and to increase the rate of lipolysis.

Additionally, there has been much focus on development of improved dairy-alternatives in recent years. Flavourings developed from the data outlined in this thesis may provide authentic cooked cheese flavour for cheese-alternatives to be used in dairy-free cooked applications such as pizzas and ready meals.

7.5 References

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Appendices

Appendix 1 Quantitation of selected odorants from cooked Cheddar cheese in a range of cooked and uncooked cheeses.

	Uncooked						Cooked					
Compound name	Cheddar	HF	MF	LF	Mozzarella	Parmesan	Cheddar	HF	MF	LF	Mozzarella	Parmesan
methanethiol	ND [a]	ND [a]	ND [a]	ND [a]	0.004 [a]	ND [a]	0.58 [d]	0.32 [b c d]	0.21 [a b c]	0.08 [a b]	0.01 [a]	0.39 [c d]
2-methyl propanal	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	5.9 [d]	3.2 [c]	2.3 [b c]	1.5 [a b]	0.15 [a]	5.9 [d]
2,3-butanedione	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	1.4 [b]	1.7 [b]	1.6 [b]	1.3 [b]	2.5 [c]	1.2 [b]
3-methyl butanal	0.12 [a]	0.04 [a]	0.05 [a]	0.06 [a]	0 [a]	0.16 [a]	33 [b c]	45 [c]	31 [b c]	16 [a b]	0.87 [a]	13 [a]
2-methyl butanal	0.18 [a]	0.15 [a]	0.12 [a]	0.28 [a]	0.03 [a]	0.2 [a]	16 [b]	3.4 [a]	2 [a]	3.1 [a]	0.5 [a]	22 [c]
dimethyl disulfide	0.004 [a]	0.02 [a]	0.01 [a]	0.03 [a]	0.1 [a]	0.01 [a]	0.4 [a b]	0.65 [a b c]	0.31 [a b]	0.86 [b c]	0.24 [a]	1.2 [c]
butanoic acid	6 [d]	4.3 [c d]	3.5 [b c d]	0.26 [a]	0.69 [a b]	40 [f]	4 [c d]	2.5 [a b c]	3 [a b c]	0.4 [a]	0.11 [a]	18.5 [e]
3-methyl butanoic acid	0.11 [e]	0.05 [b c]	0.07 [c d]	ND [a]	0.01 [a]	0.11 [e]	0.11 [d e]	0.05 [b c]	0.05 [b c]	0.01 [a b]	0.01 [a]	0.14 [e]
2-heptanone	0.22 [a]	0.19 [a]	0.16 [a]	0.04 [a]	0.04 [a]	1.45 [a]	10 [c]	12 [c]	13 [c]	1.3 [a]	5.9 [b]	5.7 [b]
methional	0.07 [a]	0.01 [a]	0.01 [a]	0.01 [a]	ND [a]	0.1 [a]	0.79 [d]	0.55 [c]	0.37 [b]	0.11 [a]	0.01 [a]	0.42 [b]
hexanoic acid	2.77 [c d]	2.5 [b c d]	3 [d]	0.43 [a]	0.87 [a b c]	50 [f]	1.5 [a b c d]	0.86 [a b c]	1.5 [a b c d]	0.58 [a b]	0.09 [a]	12 [e]
dimethyl trisulfide	ND [a]	0.85 [c]	0.22 [a]	0.01 [a]	0.01 [a]	ND [a]	0.3 [a b]	0.23 [a]	0.14 [a]	0.73 [b c]	0.09 [a]	0.89 [b c]
trimethylpyrazine	0.02 [a b]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	1.2 [d]	0.34 [c]	0.3 [c]	0.21 [a b c]	0.26 [b c]	0.16 [a b c]

4261

	Uncooked						Cooked					
Compound name	Cheddar	HF	MF	LF	Mozzarella	Parmesan	Cheddar	HF	MF	LF	Mozzarella	Parmesan
3- methyl -1,2-cyclopentanedione	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	0.21 [c]	0.09 [b]	0.06 [b]	0.01 [a]	0.01 [a]	ND [a]
phenylacetaldehyde	0.47 [a]	0.05 [a]	0.06 [a]	0.02 [a]	ND [a]	ND [a]	3.9 [c d]	4.1 [d]	4.6 [d]	2.6 [b c]	0.08 [a]	2.39 [b]
4-hydroxy-2,5-dimethyl-3(2H)-furanone	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	0.09 [d]	0.05 [c]	0.04 [b c]	0.01 [a]	0.01 [a b]	0.02 [a b]
4-methylphenol	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	0 b [c]	0.01 [e]	0 [c]	0 [a b c]	0 [b c]	0 [a b]	0.01 [d]
3-ethyl-2,5-dimethyl- pyrazine	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	0.58 [c]	0.14 [b]	0.13 [b]	0.16 [b]	0.09 [a b]	0.17 [b]

4262

4263 Quantitation of selected odorants (μg/g) from cooked Cheddar cheese in a range of cooked and uncooked cheeses. Data are means of
4264 triplicate analyses. Letters a-f indicate significance based on Tukey's HSD, where different letters indicate significant difference
4265 between the means ($p < 0.05$) . ND indicates compounds which were not detected. Data are referred to in chapter 3.

4266

Appendix 2 Quantitation of amino acid precursors to Strecker aldehydes.

	Valine	Leucine	Isoleucine	Methionine	Phenylalanine
Uncooked HF	218	484	822	24	368
Uncooked MF	262	480	815	45	386
Uncooked LF	573	935	1591	140	1022
Uncooked Cheddar	1141	1369	521	113	491
Uncooked Mozzarella	17.8	17.7	7.9	1.2	10.6
Uncooked Parmesan	2481	2178	1932	626	920

Quantitation of amino acid precursors to Strecker aldehydes (mg/kg). Data were means of triplicate analyses. Data are referred to in chapter 3.

4274

4275 **Appendix 3** Quantitation of 2-methylketones, esters, fatty acids, and pyrazines in a range of cooked and uncooked cheeses.

2-methylketones	Uncooked Cheddar	Uncooked HF	Uncooked MF	Uncooked LF	Uncooked Mozzarella	Uncooked Parmesan	Cooked Cheddar	Cooked HF	Cooked MF	Cooked LF	Cooked Mozzarella	Cooked Parmesan
2-pentanone	0.026 [a]	ND [a]	ND [a]	ND [a]	0.019 [a]	0.12 [a]	1.2 [c]	1.9 [d]	1.9 [d]	0.13 [a]	1.4 [c]	0.73 [b]
2-heptanone	0.22 [a]	0.19 [a]	0.16 [a]	0.042 [a]	0.041 [a]	1.5 [a]	10 [c]	12 [c]	13 [c]	1.3 [a]	5.9 [b]	5.7 [b]
2-nonanone	0.041 [a]	1.4 [b c]	0.82 [a b]	0.022 [a]	0.022 [a]	1.1 [a b c]	3.4 [d]	3.7 [d]	4.1 [d]	0.71 [a b]	1.2 [b c]	2 [c]
2-dodecanone	0.005 [a]	0.095 [a]	0.034 [a]	0.007 [a]	0.002 [a]	0.086 [a]	0.74 [c]	0.7 [c]	0.79 [c]	0.11 [a]	0.25 [a b]	0.4 [b]
2-undecanone	0.004 [a]	0.081 [a]	0.029 [a]	0.006 [a]	0.002 [a]	0.073 [a]	0.62 [c]	0.59 [c]	0.67 [c]	0.093 [a]	0.21 [a b]	0.34 [b]
2-tridecanone	0.001 [a]	ND [a]	ND [a]	0.003 [a]	0.001 [a]	0.015 [a]	0.24 [c]	0.25 [c]	0.26 [c]	0.053 [a b]	0.079 [a b]	0.13 [b]
Esters												
ethyl butanoate	0.78 [c]	2.6 [f]	1.7 [d]	0.24 [b]	ND [a]	2.2 [e]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]
butyl acetate	0.026 [c]	0.068 [d]	0.063 [d]	0.013 [b]	ND [a]	0.11 [e]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]
ethyl 2 methyl butanoate	0.085 [c]	0.22 [e]	0.18 [d]	0.019 [b]	ND [a]	0.24 [e]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]
ethyl 3 methyl butanoate	0.047 [b]	0.14 [d]	0.11 [c]	0.01 [a]	ND [a]	0.141 [d]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]
3 methyl 1 butanol acetate	0.19 [b]	0.6 [d]	0.48 [c]	0.051 [a]	ND [a]	0.69 [e]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]
methyl hexanoate	0.004 [a]	0.035 [b]	0.036 [b]	ND [a]	ND [a]	0.081 [c]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]
ethyl hexanoate	0.022 [a]	0.67 [c]	0.42 [b]	0.011 [a]	ND [a]	0.91 [d]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]
hexyl acetate	0.006 [a]	0.24 [c]	0.21 [c]	ND [a]	ND [a]	0.14 [b]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]
methyl octanoate	ND [a]	0.022 [c]	0.017 [b]	ND [a]	ND [a]	0.026 [d]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]
ethyl octanoate	0.003 [a]	0.058 [b]	0.041 [b]	0.012 a	ND [a]	0.3 [c]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]
ethyl decanoate	0.002 [a]	0.025 [c]	0.024 [c]	0.012 [b]	ND [a]	0.059 [d]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]

Fatty Acids												
Acetic acid	3.1 ^{b}	1.3 $^{a\,b}$	1.4 $^{a\,b}$	0.88 $^{a\,b}$	0.053 ^{a}	9.6 ^{d}	5.9 ^{c}	2.8 ^{b}	2.5 $^{a\,b}$	0.6 $^{a\,b}$	1.1 $^{a\,b}$	11 ^{d}
butanoic acid	6 ^{d}	4.3 $^{c\,d}$	3.5 $^{b\,c\,d}$	0.26 ^{a}	0.69 $^{a\,b}$	40 ^{f}	4 $^{c\,d}$	2.5 $^{a\,b\,c}$	3 $^{a\,b\,c}$	0.4 ^{a}	0.11 ^{a}	18 ^{e}
3-methyl butanoic acid	0.11 ^{e}	0.052 $^{b\,c}$	0.068 $^{c\,d}$	0 ^{a}	0.008 ^{a}	0.11 ^{e}	0.11 $^{d\,e}$	0.052 $^{b\,c}$	0.048 $^{b\,c}$	0.014 $^{a\,b}$	0.009 ^{a}	0.14 ^{e}
pentanoic acid	0.037 ^{b}	ND ^{a}	ND ^{a}	ND ^{a}	0.005 ^{a}	0.24 ^{d}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}
hexanoic acid	2.8 $^{c\,d}$	2.5 $^{b\,c\,d}$	3 ^{d}	0.43 ^{a}	0.87 $^{a\,b\,c}$	51 ^{f}	1.5 $^{a\,b\,c\,d}$	0.86 $^{a\,b\,c}$	1.5 $^{a\,b\,c\,d}$	0.58 $^{a\,b}$	0.087 ^{a}	12 ^{e}
heptanoic acid	0.013 $^{a\,b}$	ND ^{a}	ND ^{a}	ND ^{a}	0.029 ^{b}	0.43 ^{c}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}
octanoic acid	0.29 $^{a\,b}$	0.5 $^{a\,b}$	0.59 ^{b}	0.18 $^{a\,b}$	0.65 ^{b}	6.6 ^{d}	0.19 $^{a\,b}$	0.17 $^{a\,b}$	0.45 $^{a\,b}$	0.28 $^{a\,b}$	0.025 ^{a}	1.4 ^{c}
nonanoic acid	0.004 ^{a}	ND ^{a}	0.008 ^{a}	0.005 ^{a}	0.005 ^{a}	0.038 $^{a\,b}$	ND ^{a}	0.021 ^{a}	0.015 ^{a}	0.092 ^{b}	0.013 ^{a}	0.02 ^{a}
decanoic acid	0.06 ^{a}	0.16 $^{a\,b}$	0.18 $^{a\,b}$	0.054 ^{a}	0.17 $^{a\,b}$	1.8 ^{c}	0.067 ^{a}	0.065 ^{a}	0.16 $^{a\,b}$	0.076 ^{a}	ND ^{a}	0.32 ^{b}
undecanoic acid	0.005 ^{a}	0.011 ^{a}	0.011 ^{a}	0.004 ^{a}	0.006 ^{a}	0.071 ^{b}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}
Pyrazines												
methyl pyrazine	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	0.6 ^{c}	0.22 ^{b}	0.12 $^{a\,b}$	0.032 ^{a}	0.24 ^{b}	0.14 $^{a\,b}$
2,3-dimethyl-pyrazine	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	0.23 ^{c}	0.061 $^{a\,b}$	0.041 $^{a\,b}$	0.029 $^{a\,b}$	0.064 ^{b}	0.051 $^{a\,b}$
2,5-dimethyl-pyrazine	0.22 ^{a}	2.2 ^{d}	1.1 $^{a\,b}$	0.008 ^{a}	ND $^{b\,c}$	0.16 ^{a}	1.8 ^{d}	0.39 $^{a\,b}$	0.34 $^{a\,b}$	0.11 ^{a}	0.82 $^{b\,c}$	0.26 ^{a}
2-ethyl-pyrazine	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	0.088 ^{d}	0.047 ^{c}	0.03 $^{b\,c}$	0.012 $^{a\,b}$	0.037 ^{c}	0.01 $^{a\,b}$
2-ethyl-6-methyl- pyrazine	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	0.24 ^{c}	0.063 ^{b}	0.042 $^{a\,b}$	0.039 $^{a\,b}$	0.042 $^{a\,b}$	0.077 ^{b}
2-ethyl-5-methyl- pyrazine	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	ND ^{a}	0.41 ^{d}	0.16 $^{b\,c}$	0.19 $^{b\,c}$	0.11 $^{a\,b}$	0.24 ^{c}	0.03 ^{a}
trimethylpyrazine	0.02 $^{a\,b}$	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	1.2 ^{d}	0.34 ^{c}	0.3 ^{c}	0.21 $^{a\,b\,c}$	0.26 $^{b\,c}$	0.16 $^{a\,b\,c}$
3-ethyl-2,5-dimethyl- pyrazine	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	0.58 ^{c}	0.14 ^{b}	0.13 ^{b}	0.16 ^{b}	0.088 $^{a\,b}$	0.17 ^{b}

Caption: Quantitation of 2-methylketones, esters, fatty acids, and pyrazines (μg/g) in a range of cooked and uncooked cheeses. Data are means of triplicate analyses. Letters a-e indicate significance based on Tukey's HSD, where different letters indicate significant difference between the means ($p < 0.05$) . ND indicates compounds which were not detected. Data are referred to in chapter 3.

Appendix 4 Quantitation of diketopiperazines in a range of cooked and uncooked cheeses.

DKP	HF Cooked	HF uncooked	HF24 Cooked	HF24 uncooked	MF Cooked	MF uncooked	LF Cooked	LF uncooked	Mozz Cooked	Mozz uncooked	Parm Cooked	Parm uncooked	Ched Cooked	Ched uncooked
c-Val-Pro	32.7	ND	379.8	ND	44	ND	31.5	ND	33.8	7.4	153.5	6.8	512.6	3.5
c-Leu-Pro	16.1	ND	139.2	ND	27.7	ND	12.9	ND	21.5	5.5	91.3	0	273.3	0
c-Ala-Pro	20.6	ND	434.3	ND	26.2	ND	15.2	ND	ND	ND	159.8	ND	403.5	ND
c-Pro-Pro	65	ND	246	ND	68.8	ND	53.7	ND	ND	ND	32.7	ND	57.7	ND

Caption: Mean quantitation of diketopiperazines (mg/kg) in cooked and uncooked cheeses. ND indicates compounds which were not detected. Data are referred to in chapter 4.

Appendix 5 Quantitation of organic acids in a range of cooked and uncooked cheeses.

	Citric acid		Malic Acid		Lactic acid		Acetic acid		Propanoic acid	
Uncooked Cheddar	10.40	b	32.22	a	448.43	a	49.39	ab	126.57	bc
Cooked Cheddar	6.03	bcd	3.64	def	362.23	abc	83.21	a	368.84	bc
Uncooked Parmesan	1.56	d	3.07	ef	291.67	abc	47.76	b	476.93	b
Cooked Parmesan	2.08	d	4.39	cdef	346.14	abc	45.00	b	1625.37	a
Uncooked Mozzarella	9.26	bcd	13.89	b	74.26	d	39.26	b	2.31	c
Cooked Mozzarella	16.82	a	1.45	f	88.08	d	36.84	b	2.73	c
Uncooked LF	4.34	bcd	2.03	ef	198.94	cd	35.00	b	20.42	c
Cooked LF	3.50	cd	4.66	cdef	366.25	abc	48.13	b	50.85	c
Uncooked MF	3.16	cd	6.78	cde	280.85	abc	31.42	b	16.25	c
Cooked MF	5.02	bcd	8.64	c	406.39	abc	59.30	ab	47.69	c
Uncooked HF	2.54	d	6.62	cde	237.81	bcd	27.36	b	10.95	c
Cooked HF	6.13	bcd	7.90	cd	379.07	ab	44.90	b	56.20	c

Quantitation of organic acid (mg/kg) in cooked and uncooked cheeses. Data are means of triplicate analyses. Letters a-f indicate significance based on Tukey's HSD, where different letters indicate significant difference between the means ($p < 0.05$) . ND indicates compounds which were not detected. Data are referred to in chapter 4.

Appendix 6 Quantitation of sugars in a range of cooked and uncooked cheeses.

	Lactose		Glucose		Galactose	
Cheddar Cooked	0.19	d	0.0000	c	0.001	c
Cheddar uncooked	0.00	d	0.0000	c	0.000	c
HF Cooked	28.76	cd	0.0251	c	0.240	c
HF uncooked	53.15	bc	0.0919	b	0.370	c
LF Cooked	1.31	d	0.0000	c	0.069	c
LF uncooked	1.23	d	0.0000	c	0.156	c
MF Cooked	7.24	d	0.1195	ab	0.456	c
MF uncooked	7.96	d	0.1396	ab	0.452	c
Mozzarella Cooked	66.73	d	0.0000	c	18.337	b
Mozzarella uncooked	157.14	a	0.0000	c	40.000	a
Parmesan Cooked	0.06	d	0.0000	c	0.000	c
Parmesan uncooked	0.00	d	0.0162	c	0.002	c

Quantitation of sugars (mg/ kg) in cooked and uncooked cheeses. Data are means of triplicate analyses. Letters a-d indicate significance based on Tukey's HSD, where different letters indicate significant difference between the means ($p < 0.05$) . Data are referred to in chapter 4.

Appendix 7 Quantitation of amino acids in a range of cooked and uncooked cheeses.

	Ala		Gly		Val		Leu		Ile		Thr		Ser		Pro		Asn	
3M HF	76.5	F	42.9	E	217.7	FG	484.1	EFG	821.7	EF	35.6	EF	65.5	FG	71.7	EFG	187.1	DEFGH
3M MF	75.0	F	37.9	E	262.0	FG	479.6	EFG	815.3	EF	30.9	EF	38.9	G	63.0	EFG	144.0	EFGH
3M LF	290.4	BCD	173.7	B	572.6	CDEF	935.3	CDE	1591.2	CDEF	144.4	CDE	149.3	CDEFG	252.4	BCD	478.9	ABC
6M HF	97.4	F	42.9	E	274.1	EFG	634.5	DEFG	1075.8	DEF	55.0	EF	78.6	EFG	56.7	FG	215.1	CDEFGH
6M MF	90.9	F	37.9	E	263.7	FG	607.9	EFG	1031.2	DEF	46.9	EF	57.1	FG	60.1	EFG	207.6	DEFGH
6M LF	184.8	CDEF	90.1	CDE	549.8	CDEF	853.7	CDEFG	1447.0	CDEF	128.2	CDEF	149.3	CDEFG	170.1	BCDEFG	403.6	ABCDE
9M HF	139.3	DEF	70.6	DE	398.8	DEFG	862.5	CDEFG	614.3	F	71.6	EF	99.3	EFG	108.7	DEFG	344.9	BCDEFG
9M MF	130.0	EF	52.1	E	382.4	EFG	823.6	CDEFG	1004.5	DEF	69.0	EF	97.2	EFG	105.7	DEFG	298.9	CDEFGH
9M LF	177.4	CDEF	92.5	BCDE	518.0	CDEFG	829.1	CDEFG	1407.7	CDEF	124.7	CDEF	139.6	CDEFG	173.8	BCDEFG	396.5	ABCDEF
12M HF	121.2	EF	73.4	DE	468.7	DEFG	842.9	CDEFG	1430.8	CDEF	90.5	DEF	108.0	DEFG	152.5	BCDEFG	263.6	CDEFGH
12M MF	155.6	DEF	82.6	CDE	450.6	DEFG	902.0	CDEF	1165.2	DEF	96.4	DEF	139.9	CDEFG	127.8	CDEFG	324.8	BCDEFGH
12M LF	319.6	BC	158.1	BC	914.2	BC	1472.1	BC	2498.4	ABCD	228.2	BC	260.3	BCDE	302.9	BC	576.5	AB
18M HF	256.0	BCDE	150.6	BCD	803.4	BCD	1454.8	BC	2467.6	ABCD	202.2	BCD	299.6	BCD	222.2	BCDEF	443.7	ABCD
18M MF	217.2	BCDEF	106.9	BCDE	722.0	BCDE	1359.5	BCD	2306.6	BCDE	154.2	BCDE	242.1	BCDEF	173.1	BCDEFG	389.9	ABCDEF
18M LF	566.8	A	299.8	A	1613.4	A	2380.7	A	4040.4	A	468.8	A	634.4	A	564.3	A	637.2	A
24M HF	288.4	BCD	184.2	B	920.1	BC	1726.7	AB	2826.6	ABC	228.9	BC	322.2	BC	234.6	BCDE	414.6	ABCDE
24M MF	345.3	B	179.4	B	1062.3	B	1743.3	AB	2959.5	ABC	273.3	B	410.2	B	325.2	B	431.2	ABCDE
24M LF	542.9	A	294.8	A	1533.1	A	2289.9	A	3896.5	AB	467.5	A	640.3	A	573.2	A	288.5	CDEFGH
C 3M HF	64.5	F	32.9	E	81.5	G	218.2	FG	371.9	F	16.6	F	31.5	G	47.1	FG	84.5	GH
C 3M MF	63.6	F	25.4	E	72.6	G	171.7	G	293.2	F	15.2	F	23.7	G	40.5	G	73.0	H
C 3M LF	99.1	F	44.1	E	132.6	G	228.0	FG	388.3	F	36.7	EF	50.1	G	76.2	DEFG	129.3	FGH

	Asp		Met		Glu		Phe		Orn		Lys		His		Tyr		Trp	
3M HF	69.5	C	24.5	D	230.8	DE	367.5	GHIJ	76.2	E	167.1	DEF	26.6	E	82.9	FGH	4.2	DE
3M MF	51.6	C	45.3	D	344.7	DE	386.5	FGHIJ	62.3	E	106.5	DEF	3.4	E	74.8	FGH	6.5	DE
3M LF	178.7	C	139.6	CD	1021.9	CDE	830.3	CDEF	345.2	BC	635.0	BC	45.5	DE	178.6	EFG	14.1	CDE
6M HF	82.2	C	39.7	D	346.9	DE	391.3	FGHIJ	102.3	E	117.7	DEF	0.0	E	124.2	FGH	1.2	E
6M MF	89.7	C	61.3	D	415.3	DE	384.7	FGHIJ	42.1	E	38.8	F	0.0	E	60.6	GH	0.0	E
6M LF	164.1	C	99.8	CD	1042.4	CDE	645.6	CDEFGHI	135.3	CDE	154.8	DEF	0.0	E	137.6	FGH	6.4	DE
9M HF	113.7	C	148.3	CD	523.4	DE	571.5	DEFGHIJ	74.3	E	110.8	DEF	0.0	E	151.8	FGH	6.4	DE
9M MF	131.9	C	89.2	CD	495.6	DE	507.7	EFGHIJ	103.5	E	147.3	DEF	0.0	E	135.4	FGH	2.1	DE
9M LF	144.3	C	168.9	CD	824.2	CDE	647.3	CDEFGH	163.1	BCDE	202.1	CDEF	0.0	E	159.6	EFGH	12.1	CDE
12M HF	126.9	C	142.6	CD	1127.8	CDE	542.0	EFGHIJ	124.9	DE	236.7	CDEF	34.7	E	219.7	DEF	18.4	CDE
12M MF	139.4	C	106.4	CD	610.4	CDE	560.7	DEFGHIJ	113.3	DE	178.3	DEF	23.4	E	166.4	EFGH	7.2	DE
12M LF	349.9	BC	359.0	B	1891.2	BC	1041.4	BC	354.7	B	490.4	BCD	54.7	DE	302.7	CDE	45.2	BC
18M HF	290.0	BC	239.3	BC	1842.7	BC	851.7	CDE	225.1	BCDE	501.1	BCD	180.8	BC	352.0	BCD	34.7	CD
18M MF	277.0	BC	141.5	CD	1415.5	BCD	761.5	CDEFG	192.2	BCDE	461.7	BCDE	128.2	CD	302.2	CDE	11.3	DE
18M LF	981.2	A	646.9	A	3831.5	A	1552.3	A	646.6	A	1219.2	A	315.1	A	502.3	A	91.6	A
24M HF	252.1	BC	372.5	B	1873.0	BC	1000.2	BCD	198.5	BCDE	470.7	BCDE	228.3	B	457.3	AB	68.9	AB
24M MF	559.3	B	400.3	B	2468.0	B	1005.4	BCD	320.4	BCD	761.4	B	224.8	B	401.1	ABC	44.8	BC
24M LF	1259.7	A	663.6	A	4133.0	A	1396.9	AB	615.1	A	1295.2	A	368.8	A	501.6	A	96.8	A
C 3M HF	41.7	C	28.1	D	67.6	E	192.4	IJ	44.5	E	50.9	F	4.5	E	35.7	GH	4.5	DE
C 3M MF	33.9	C	21.3	D	113.4	E	163.4	J	59.2	E	66.9	EF	5.3	E	29.4	H	2.3	DE
C 3M LF	59.5	C	42.7	D	206.4	DE	226.2	HIJ	81.0	E	127.9	DEF	17.4	E	45.9	GH	6.4	DE

Quantitation of amino acids (mg/kg) in cooked and uncooked cheeses. Data are means of triplicate analyses. 3M = 3 months aged, 6M = 6 months aged, 9M = 9 months aged, 12M = 12 months aged, 24M = 24 months aged, C 3M = cooked 3 months aged. LF, MF and HF = low, mediam and high fat Cheddar respectively. Letters A-J indicate significance based on Tukey's HSD, where different letters indicate significant difference between the means ($p < 0.05$) . Data are referred to in chapter 4.

4304 **Appendix 8** Quantitation of γ -Glutamyl peptides in a range of cooked and uncooked cheeses.

	y-Glu-Glu	y-Glu-Val	y-Glu-Met	y-Glu-Tyr	y-Glu-Leu	y-Glu-Phe
3M HF	0.000 e	0.000 e	2.1	0.000 f	0.014 i	0.005 f
3M MF	0.000 e	0.000 e	2.0	0.000 f	0.016 i	0.006 f
3M LF	0.004 de	0.001 e	11.6	0.000 f	0.026 i	0.011 f
6M HF	0.000 e	0.001 e	1.4	0.000 f	0.021 i	0.007 f
6M MF	0.000 e	0.002 e	1.9	0.000 f	0.024 i	0.007 f
6M LF	0.000 e	0.012 e	4.7	0.003 ef	0.043 hi	0.013 f
9M HF	0.000 e	0.003 e	2.7	0.000 f	0.022 i	0.007 f
9M MF	0.000 e	0.005 e	2.6	0.000 f	0.023 i	0.007 f
9M LF	0.000 e	0.012 e	7.0	0.002 f	0.046 ghi	0.013 f
12M HF	0.045 c	0.060 cd	82.1	0.038 d	0.245 ef	0.128 de
12M MF	0.000 e	0.016 de	44.5	0.010 ef	0.087 ghi	0.038 f
12M LF	0.000 e	0.037 cde	80.4	0.019 e	0.155 fgh	0.078 ef
18M HF	0.044 c	0.069 c	138.4	0.048 cd	0.319 de	0.173 cd
18M MF	0.019 d	0.039 cde	61.1	0.018 e	0.168 fg	0.073 ef
18M LF	0.058 bc	0.115 b	218.4	0.055 bc	0.405 cd	0.235 c
24M HF	0.072 ab	0.122 b	260.3	0.095 a	0.577 b	0.318 b
24M MF	0.045 c	0.124 b	166.8	0.066 b	0.472 bc	0.238 c
24M LF	0.087 a	0.202 a	379.3	0.096 a	0.710 a	0.442 a
C 3M LF	0.000 e	0.000 e	3.6	0.000 f	0.011 i	0.004 f
C 3M MF	0.000 e	0.000 e	0.9	0.000 f	0.009 i	0.004 f
C 3M HF	0.000 e	0.000 e	0.7	0.000 f	0.008 i	0.003 f

Quantitation of γ-glutamyl peptides (mg/kg) in cooked and uncooked cheeses. 3M = 3 months aged, 6M = 6 months aged, 9M = 9 months aged, 12M = 12 months aged, 24M = 24 months aged, C 3M = cooked 3 months aged. LF, MF and HF = low, mediam and high fat Cheddar respectively. Data are means of triplicate analyses. Letters a- i indicate significance based on Tukey's HSD, where different letters indicate significant difference between the means ($p < 0.05$). Data are referred to in chapter 4.

Appendix 9

Quantitation of compounds (mg/kg) in cooked and uncooked Cheddars (wet weight).

	HFC	MFC	LFC	HFR	MFR	LFR
2-pentanone	0.275 a	0.240 a	0.050 b	0.035 b	0.040 b	0.011 b
2-heptanone	1.39 a	0.992 a	0.071 b	0.042 b	0.038 b	0.030 b
2-nonanone	1.70 a	1.89 a	0.035 b	0.030 b	0.029 b	0.014 b
2-undecanone	2.78 a	2.26 b	0.021 c	0.036 c	0.030 c	0.006 c
2-tridecanone	4.99 a	3.15 b	0.043 c	0.070 c	0.051 c	0.010 c
2-pentadecanone	4.21 a	2.13 ab	0.076 b	0.064 b	0.034 b	0.014 b
acetic acid	26.6 c	63.8 a	57.4 ab	23.1 c	32.2 bc	39.6 abc
propanoic acid	0.625 a	1.11 a	0.253 a	1.26 a	0.850 a	0.114 a
Butanoic acid	3.90 b	10.8 a	2.51 b	11.8 a	12.1 a	3.97 b
pentanoic acid	0.111 a	0.133 a	0.045 b	0.147 a	0.141 a	0.057 b
hexanoic acid	1.88 c	4.84 ab	1.76 c	5.34 a	5.97 a	3.52 b
heptanoic acid	0.252 a	0.193 ab	0.059 c	0.188 ab	0.179 ab	0.105 bc
octanoic acid	3.44 bc	5.14 ab	1.66 c	7.15 a	7.11 a	4.28 b
nonanoic acid	1.15 a	0.75 ab	0.342 b	0.752 ab	0.467 ab	0.414 b
decanoic acid	5.87 abc	5.40 bc	1.24 c	10.8 a	8.50 ab	2.65 c

dodecanoic acid	2.47 a	2.87 a	0.395 a	4.24 a	2.95 a	0.544 a
dimethyl disulfide	0.110 b	0.074 bc	0.214 a	ND c	0.004 c	0.007 c
dimethyl trisulfide	0.078 b	0.078 b	0.132 a	ND c	0.004 c	0.004 c
dimethyl sulfone	0.283 a	0.239 a	0.271 a	0.249 a	0.215 a	0.246 a
hexanal	0.091 a	0.035 ab	ND b	0.021 ab	ND b	0.012 ab
heptanal	0.102 a	0.005 b	ND b	0.024 ab	0.005 b	0.003 b
nonanal	0.543 a	0.028 b	0.047 b	0.230 ab	0.068 b	0.047 b
undecanal	0.447 a	0.108 a	ND a	ND a	ND a	0.008 a
dodecanal	0.300 a	0.395 a	ND a	0.022 a	ND a	0.015 a
(E)-2-undecenal	0.024 a	0.011 ab	ND b	ND b	ND b	ND b
(E)-2-decenal	0.035 a	0.018 ab	ND b	ND b	ND b	ND b
(E,E)-2,4 decadienal	0.198 a	0.044 b	0.001 b	ND b	0.001 b	ND b
3-methylbutanal	0.220 a	0.309 a	0.113 a	ND a	ND a	ND a
benzeneacetaldehyde	1.03 a	1.19 a	0.346 b	0.001 c	ND c	0.010 c
ethyl acetate	28.2 a	35.3 a	3.47 a	13.3 a	11.5 a	5.05 a
ethyl butanoate	0.042 a	0.050 a	0.099 a	0.132 a	0.105 a	0.028 a
ethyl hexanoate	0.021 b	0.011 b	0.012 b	0.082 a	0.048 ab	0.009 b

ethyl decanoate	0.007 bc	0.015 ab	ND c	0.017 ab	0.029 a	0.001 c
4-methyl-2-phenyl-2-pentenal	0.005 ab	0.007 a	0.002 bc	ND c	ND c	ND c
2-phenyl-2-butenal	0.061 a	0.034 b	0.070 a	ND c	ND c	ND c
5-methyl-2-phenyl-2-hexenal	0.076 a	0.035 b	0.072 a	ND c	ND c	ND c
5-methyl-2- isopropyl-2-hexenal	0.132 b	0.277 a	0.059 b	0.010 b	ND b	0.006 b
2-methyl-2-pentenal	0.898 a	0.555 a	0.065 a	0.551 a	0.191 a	0.004 a
1-ethylidene-3-methyl-butanal	0.014 b	0.031 a	0.006 b	0.000 b	0.000 b	0.000 b
3-hexen-2-one	10.373 a	13.666 a	1.121 a	12.111 a	4.067 a	0.006 a
2-methyl-2-butenal	0.047 a	0.037 ab	0.008 ab	0.021 ab	0.015 ab	0.002 b
2-hydroxy-3-methyl-2-cyclopenten-1-one,	0.328 a	0.127 a	0.028 a	0.012 a	0.042 a	0.006 a
cyclotene	0.136 b	0.222 a	0.058 c	0.019 c	0.016 c	0.004 c
2,3-dihydro-3,5-dihydro-4*H*-pyran-4-one	5.63 a	0.292 b	0.594 b	0.248 b	0.164 b	0.079 b
maltol	1.68 a	0.207 b	0.064 b	0.005 b	0.003 b	0.007 b
4,5-dimethyl-2-isobutyl-1,3-dioxolane (1)	0.019 a	0.052 a	0.078 a	0.000 a	0.000 a	0.001 a
4,5-dimethyl-2-isobutyl-1,3-dioxolane (2)	0.000 b	0.000 b	0.040 a	0.000 b	0.006 b	0.000 b
4,5-dimethyl-2-(phenylmethyl)-1,3-dioxolane	0.000 a	0.062 a	0.048 a	0.000 a	0.000 a	0.000 a
2,4,5-trimethyl-1,3-dioxolane (1)	2.875 b	10.472 a	0.601 b	0.535 b	2.444 b	0.253 b
2,4,5-trimethyl-1,3-dioxolane (3)	0.367 a	0.647 a	0.000 a	0.021 a	0.017 a	0.000 a

2,4,5-trimethyl-1,3-dioxolane (2)	2.347 a	4.211 a	0.169 a	0.117 a	0.874 a	0.065 a
4,5-dimethyl-2-(phenylmethyl)-1,3-dioxolane	0.266 b	0.922 a	0.383 b	0.003 b	0.007 b	0.007 b
2,4,5-trimethyl-2-heptyl-1,3-dioxolane	0.145 ab	0.378 a	0.005 b	0.000 b	0.000 b	0.000 b
2 acetylpyrrole	0.149 a	0.168 a	0.054 ab	0.004 b	ND b	ND b
2-furfural	0.073 a	0.080 a	0.010 b	ND b	0.001 b	ND b
γ octalactone	0.106 a	ND b	ND b	0.029 b	0.010 b	0.002 b
δ decalactone	2.06 a	1.62 ab	0.257 b	1.25 ab	1.19 ab	0.161 b
acetoin	0.232 c	0.644 bc	2.00 b	0.283 c	0.883 bc	4.05 a
2,3 butanediol	4.39 c	4.48 c	11.7 ab	5.30 bc	5.48 bc	13.8 a
1-methyl-2-pyrrolidinone	0.539 a	0.001 a	0.003 a	0.348 a	0.145 a	0.053 a
3 methylbutanol	0.194 a	0.176 a	0.030 a	1.73 a	0.790 a	0.138 a
p-cresol	0.041 b	0.056 ab	0.071 a	0.007 c	0.006 c	0.006 c
phenylacetic acid	0.156 a	0.184 a	0.115 a	0.058 a	0.050 a	0.080 a
phenylethanol	0.065 a	0.340 a	0.024 a	0.600 a	0.276 a	0.038 a

Data are means of triplicate analyses. Letters a-c indicate significance based on Tukey's HSD, where different letters indicate significant difference between the means ($p < 0.05$). ND indicates compounds which were not detected. Data are referred to in chapter 6

4316 **Appendix 10** Mass Spectra and Linear Retention Indices of Synthesized dioxolanes.

Synthesised compound	LRI (FFAP)[a]	LRI (DB5)[b]	Fragmentation [c]
2,4,5-trimethyl-1,3-dioxolane (1)	990	747	101 (100), 43 (71), 44 (57), 73 (51), 72 (49), 55 (35), 45 (27), **115** (16), 57 (12), 102 (6), 71 (5), 41 (4), 74 (2), 42 (2)
2,4,5-trimethyl-1,3-dioxolane (2)	1017	761	101 (100), 43 (54), 73 (44), 44 (44), 55 (39), 72 (37), 45 (24), 57 (10), **115** (7), 102 (6), 71 (4), 41 (4), 56 (2), 74 (2)
2,4,5-trimethyl-1,3-dioxolane (3)	950	719	101 (100), 43 (69), 44 (59), 72 (53), 55 (44), 73 (40), 45 (27), **115** (12), 57 (11), 102 (6), 71 (5), 41 (4), 56 (3), 42 (3)
4,5-dimethyl-2-ethyl-1,3-dioxolane (1)	1073	848	101 (100), 73 (51), 55 (36), 58 (35), 57 (29), 86 (23), 43 (18), 45 (13), 41 (7), **129** (7), 42 (7), 102 (6), 71 (6), 59 (4)
4,5-dimethyl-2-ethyl-1,3-dioxolane (2)	1102	859	101 (100), 73 (46), 55 (37), 58 (24), 57 (17), 86 (16), 43 (15), 45 (11), 102 (6), 41 (6), 42 (5), 71 (4), 59 (3), **129** (3)
4,5-dimethyl-2-ethyl-1,3-dioxolane (3)	1024	817	101 (100), 55 (40), 73 (38), 58 (32), 57 (23), 86 (22), 43 (16), 45 (12), 41 (7), 42 (6), 102 (6), **129** (6), 71 (5), 59 (4)
4,5-dimethyl-2-isopropyl-1,3-dioxolane (1)	1107	909	101 (100), 73 (42), 55 (31), 56 (28), 43 (15), 100 (8), 41 (8), 57 (7), 102 (6), 45 (5), 71 (5), **143** (4), 74 (2), 72 (2)
4,5-dimethyl-2-isopropyl-1,3-dioxolane (2)	1125	917	101 (100), 73 (41), 55 (32), 56 (22), 43 (13), 41 (6), 102 (6), 57 (6), 100 (6), 45 (5), 71 (3), 74 (2), **143** (1), 72 (1)
4,5-dimethyl-2-isopropyl-1,3-dioxolane (3)	1048	872	101 (100), 55 (32), 73 (32), 56 (28), 43 (13), 100 (9), 41 (7), 57 (7), 102 (6), 45 (5), 71 (4), **143** (3), 72 (2), 74 (1)
4,5-dimethyl-2-isobutyl-1,3-dioxolane (1)	1173	1004	101 (100), 73 (32), 55 (20), 99 (16), 43 (11), 71 (10), 102 (6), 57 (5), 41 (5), 45 (4), **157** (4), 114 (4), 85 (3), 69 (2)
4,5-dimethyl-2-isobutyl-1,3-dioxolane (2)	1202	1016	101 (100), 73 (31), 55 (20), 99 (13), 43 (9), 71 (8), 102 (5), 41 (4), 45 (4), 57 (3), 114 (3), 69 (2), 85 (2), **157** (2)
4,5-dimethyl-2-isobutyl-1,3-dioxolane (3)	1129	972	101 (100), 73 (24), 55 (22), 99 (16), 43 (10), 71 (9), 102 (6), 41 (4), 45 (4), 114 (4), 57 (4), **157** (3), 85 (2), 69 (2)
4,5-dimethyl-2-[2-(methylthio)ethyl]-1,3-dioxolane (1)	1711	1262	101 (100), 73 (90), 75 (73), 55 (63), 128 (53), 56 (48), 61 (44), 72 (32), 43 (27), **176** (27), 105 (21), 45 (18), 71 (15), 104 (13)

4,5-dimethyl-2-[2-(methylthio)ethyl]-1,3-dioxolane (2)	1746	1276	101 (100), 73 (84), 55 (63), 75 (49), 72 (38), 128 (36), 61 (32), 43 (25), 56 (25), 105 (20), 45 (15), **176** (15), 71 (12), 104 (11)
4,5-dimethyl-2-[2-(methylthio)ethyl]-1,3-dioxolane (3)	1654	1222	101 (100), 55 (68), 73 (67), 75 (57), 128 (48), 61 (38), 56 (32), 43 (26), **176** (24), 72 (21), 45 (17), 105 (14), 74 (13), 44 (6)
4,5-dimethyl-2-(phenylmethyl)-1,3-dioxolane (1)	1895	1388	101 (100), 73 (36), 55 (23), 91 (22), 119 (12), 105 (10), 102 (6), 43 (6), 103 (6), 104 (5), 65 (5), 77 (4), 78 (3), 92 (3)
4,5-dimethyl-2-(phenylmethyl)-1,3-dioxolane (2)	1918	1391	101 (100), 73 (36), 55 (24), 91 (22), 119 (10), 105 (9), 102 (6), 43 (6), 65 (5), 103 (5), 104 (4), 77 (4), 92 (3), 45 (3)
4,5-dimethyl-2-(phenylmethyl)-1,3-dioxolane (3)	1827	1347	101 (100), 73 (29), 55 (25), 91 (21), 119 (12), 105 (12), 102 (6), 103 (5), 43 (5), 104 (5), 65 (5), 77 (4), 78 (3), 92 (3)
2,2,4,5-tetramethyl-1,3-dioxolane (1)	1003	796	115 (100), 43 (97), 73 (49), 58 (44), 59 (23), 86 (20), 55 (19), 45 (16), 42 (15), 41 (9), 116 (7), 57 (5), 44 (5), 71 (3)
2,2,4,5-tetramethyl-1,3-dioxolane (2)	958	745	43 (100), 115 (94), 58 (54), 86 (31), 73 (30), 55 (22), 59 (21), 45 (18), 42 (17), 41 (9), 116 (6), 44 (5), 57 (5), 71 (3)
2,4,5-trimethyl-2-pentyl-1,3-dioxolane (1)	1366	1173	115 (100), 43 (36), 73 (22), 99 (21), 171 (17), 71 (11), 55 (11), 116 (7), 86 (5), 41 (4), 56 (3), 45 (3), 58 (3), 72 (2)
2,4,5-trimethyl-2-pentyl-1,3-dioxolane (2)	1284	1124	115 (100), 43 (33), 99 (22), 171 (14), 55 (12), 73 (11), 71 (10), 86 (7), 116 (7), 41 (4), 56 (3), 142 (3), 58 (3), 45 (3)
2,4,5-trimethyl-2-heptyl-1,3-dioxolane (1)	1772	1572	115 (100), 43 (20), 227 (17), 73 (14), 55 (8), 116 (7), 99 (5), 71 (5), 41 (4), 86 (3), 57 (3), 228 (3), 85 (2), 69 (2)
2,4,5-trimethyl-2-heptyl-1,3-dioxolane (2)	1687	1516	115 (100), 43 (19), 227 (15), 55 (8), 73 (7), 116 (7), 99 (7), 71 (5), 86 (4), 41 (3), 57 (3), 228 (2), 85 (2), 58 (2)

Numbers 1-3 in parentheses indicate isomers of each compound, which are numbered by peak area.

[a] Linear retention index on FFAP-ms column. [b] Linear retention index on an DB-5 column. [c] Ions from mass spectra in order of intensity, numbers in parentheses indicate intensity relative to base peak; molecular ion – H^+ in bold type. Data are referred to in chapter 6.

Appendix 11 Mass Spectra and Linear Retention Indices of Synthesized aldol condensation products.

Synthesised compound	LRI (FFAP)[a]	LRI (DB5)[b]	Fragmentation [c]
2-butenal	1050	<700	Wiley[d]
1-ethylidene-3-methylbutanal	1185	878	112 (100), 41 (65), 97 (64), 55 (61), 83 (56), 43 (38), 69 (36), 79 (26), 40 (25), 44 (18), 67 (17), 77 (12), 53 (12), 42 (11)
alpha-ethylidene-benzeneacetaldehyde	1936	1270	NIST[e]
3-penten-2-one	1135	ND	Wiley[d]
3-ethylidene-2-heptanone	1419	1437	Wiley[d]
3-ethylidene-2-undecanone	1827	1482	44 (100), 40 (91), 43 (56), 181 (28), 55 (26), 83 (24), 69 (21), 41 (20), 97 (20), 125 (14), 99 (12), 71 (11), 67 (11), 81 (11)
2-methyl-2-pentenal	1165	827	NIST[e]
2-methyl-2-butenal	1054	737	NIST[e]
3-hexen-2-one		848	NIST[e]
2,4-dimethyl-2-pentenal	1174	880	Wiley[d]
4-methyl-2-(1-methylethyl)-2-pentenal	1301	996	84 (100), 111 (53), 43 (52), 56 (50), 55 (47), 83 (46), 41 (38), 71 (16), 108 (15), 69 (12), 93 (10), 53 (10), 97 (9), 126 (8)
3-propylidene-2-heptanone	1461	1157	NIST[e]
3-propylidene-2-undecanone	1862	1547	111 (100), 43 (85), 181 (77), 97 (56), 55 (54), 210 (44), 69 (33), 83 (28), 67 (25), 123 (24), 57 (23), 195 (23), 81 (22), 41 (22)

4-methyl-2-(1-methylethyl)-2-pentenal	1233	988	125 (100), 55 (62), 69 (45), 41 (37), 43 (37), 107 (36), 85 (30), 97 (25), 83 (24), 79 (16), 67 (14), 56 (12), 53 (11), 91 (11)
4-methyl-2-[(methylthio)methyl]-2-pentenal	1710	1212	Wiley[d]
4-methyl-2-phenyl-2-pentenal	1946	1372	NIST[e]
3-isobutylidene-2-heptanone	1595	1182	125 (100), 43 (63), 69 (35), 168 (17), 107 (17), 41 (15), 55 (15), 81 (10), 126 (10), 67 (9), 83 (8), 97 (8), 79 (7), 111 (7)
2-isopropyl-5-methyl-2-hexenal	1367	1104	Wiley[d]
5-methyl-2-[(methylthio)methyl]-2-hexenal	1850	1335	109 (100), 81 (72), 124 (40), 79 (37), 55 (31), 41 (27), 53 (23), 43 (20), 95 (18), 172 (18), 91 (17), 77 (17), 67 (15), 48 (15)
5-methyl-2-phenyl-2-hexenal	2074	1487	NIST[e]
6-methyl-3-hepten-2-one	1340	999	Wiley[d]
3-isopentylidene-2-heptanone	1566	1293	43 (100), 125 (89), 182 (57), 167 (55), 139 (49), 55 (38), 97 (32), 69 (32), 83 (31), 111 (30), 121 (27), 85 (26), 41 (22), 81 (18)
3-isopentylidene-2-undecanone	1958	1678	43 (100), 181 (84), 139 (68), 223 (45), 238 (42), 69 (39), 85 (38), 125 (38), 97 (33), 121 (32), 111 (32), 55 (30), 83 (28), 123 (28)
2,4-diphenyl-2-butenal	2855	1926	115 (100), 222 (71), 91 (54), 103 (47), 221 (44), 178 (36), 193 (34), 116 (22), 131 (20), 89 (18), 165 (18), 191 (17), 65 (16), 179 (16)

[a] Linear retention index on FFAP-ms column. [b] Linear retention index on an DB-5 column. [c] Ions from mass spectra in order of intensity, numbers in parentheses indicate intensity relative to base peak; molecular ion – H^+ in bold type. [d] Spectral data already characterized by John Wiley & Sons, Inc (9th edition, W9N08). [e] Spectral data already characterized by NIST database (2014). Data are referred to in chapter 6.

Appendix 12 Compositional analysis of high, medium and low fat mild cheddars after 3 months ripening

Cheese	% Fat [a]	% Protein [b]	% Moisture [c]	% Ash [d]	% CHO [e]	pH [f]
High Fat	35.0	24.1	33.0	3.04	4.86	5.19
Medium Fat	26.5	27.6	35.6	3.47	6.83	5.10
Low Fat	2.0	42.5	40.1	4.91	10.49	5.31

[a] – Fat content was measured by the Gerber Technique (Badertscher et al., 2007) as described by Grandison and Ford (1986) . [b] – Protein content was measured by the Kjeldahl Technique (Kieldahl, 1883) as described in ISO 17837:2008. [c] – Moisture content was measured by loss of weight upon oven drying at 100 ◦ C. [d] – Ash content was measured by remaining weight after heating at 800 ◦ C to constant weight. [e] – Carbohydrate content was indirectly determined by the difference between the total weight and the other values. f – pH was measured by potentiometric test on 10 % slurries of cheese in deionised water.

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